EXPERIMENTAL CELL RESEARCH

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RADIATION EFFECTS OF THYMIDINE-2-C^{14}

I. UPTAKE OF THYMIDINE-2-C^{14} AND THYMINE-2-C^{14} IN THE ONION ROOT TIP[1]

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Received February 3, 1956

Since thymidine is a precursor of DNA thymine [25, 8, 10] and becomes a part of the DNA structure without diversion to any known cytoplasmic component, including RNA, a unique system is available for provoking intranuclear damage. Thymine, on the other hand, is not incorporated into DNA [24]. Radioactive thymine enters the cell without becoming a part of DNA and thus can provide a source of radiation outside the DNA molecule.

In this paper the uptake of thymidine-C^{14} by the meristematic cells of the onion root tip and its incorporation into DNA is studied. In Paper II [20] chromosome aberrations caused by thymidine-C^{14} are described.

The general problem of effects of unstable isotope atoms as a part of the genetic material has been considered by Kalckar [17], Kamen [18] and King [19]. Giles [12, 13] found that chromosome aberrations in the mitosis of *Tradescantia* microspore nuclei caused by beta radiations from both carbon-14 and phosphorus-32 were cytologically indistinguishable from those caused by external radiation such as X-rays. Ehrenberg *et al.* [6] found that onion roots treated for 3 days with P^{32} at a concentration of 0.025 μC per ml showed evidence of chromosome breakage. Beal and Scully [3] after growing onion sets in an atmosphere containing $C^{14}O_2$ for periods of 7 to 15 weeks found chromosome and chromatid breaks, fragments, bridges and micronuclei in root tips from the bulbs.

Arnason [1, 2] suggested that the mutagenic effects of phosphorus-32 would be enhanced by its inclusion in the nuclei acids and nucleoproteins of chromosomes. He emphasized the possible effects not only of beta particles emitted from within the chromatin material but also the effects of recoil and transmutation at points of decay.

[1] This work was supported in part by grants from the American Cancer Society. The authors wish to express their thanks to Dr. Martin D. Kamen for his constant interest and advice.

The work of Zirkle and Bloom [30] is unique in that protons from an external source were directed to a particular cellular locality. They found that a few dozen protons directed to the chromosomes of amphibian heart cells produced aberrations, while a few thousand particles directed to extrachromosomal regions produced no detectable effects. Hershey *et al.* [14], studying the viability of P^{32} labelled bacteriophage, where phosphorus is chiefly the phosphorus of DNA, concluded that death of the phage was brought about largely by the change $P^{32} \rightarrow S^{32}$ rather than by beta irradiation. These findings have been confirmed and amplified by Stent [28]. Lethality of P^{32} incorporated by *Amoeba proteus* has been described by Friedrich-Freksa and Kaudewitz [11].

MATERIALS AND METHODS

Thymine-C^{14} and thymidine-C^{14}.—Thymine-2-C^{14} (3.0 microcuries per micromole) was obtained as a generous gift from the Southern Research Institute. By enzymatic reaction with deoxyribose-1-phosphate the pyrimidine base was converted to thymidine. The deoxyriboside was freed of unreacted thymine by passage in 0.1*M* ammonium acetate buffer, pH 8.93, over a Dowex 1 ion exchange column [9]. The thymidine thus obtained had a specific activity of 3.0 microcuries per micromole.

Seed germination.—Seeds of *Allium cepa* (var. Sweet Spanish Utah) were chosen rather than bulbs because the roots were easily treated in the limited amounts of radioactive material available. Furthermore, the cytology of the root meristem of the onion seedling is well known and the meristem is eminently suitable for radiation studies [27, 4]. Since the literature [22] indicates that old onion seeds normally have a higher aberration rate than young seeds (we have confirmed this)recently harvested seeds were used.

Seeds were germinated on filter paper moistened with boiled tap water in sterile Petri dishes. During germination, plates were kept in the dark at 23°C. Seedlings germinating between 42 to 48 hours after plating were selected; all others were discarded. This yielded the largest uniform population. The use of chemical disinfectants was unnecessary. The selected seedlings were allowed to grow according to Schedule A: Pre-treatment growth time + treatment growth time $\cong$ 40 hours. (For example, selected roots (about 7 mm long) prior to a 19 hour treatment in radioactive material had grown for 21 hours after germination.)

Seedlings grown and treated according to this protocol yielded smears with consistently high numbers of mitotic figures. Since it became necessary to replenish our supply of seed, a new lot was obtained for those experiments carried out in 1955.

Seedling treatment.—Roots were treated on a double layer of Whatman No. 1 filter paper moistened with 0.8 ml of radioactive solution in a 20 ml Griffin beaker. Six roots were treated per beaker. The beakers were kept in a moist chamber in the dark at 23°C. The roots were observed twice daily to make certain they were in contact with the paper along their full lengths.

Fixing, staining, smearing.—After three rinsings in distilled water the root tips were blotted and fixed in freshly prepared and chilled alcohol–glacial acetic acid (3:1). A fixing time of 4–72 hours (depending on the experiment) was usual. Roots in fixative were kept in the refrigerator. The Feulgen technique [21, 5] was used for staining (hydrolysis in 1*N* HCl for 10 minutes at 58°).

Determination of radioactivity in smears.—A quantitative procedure was devised to determine the amount of C^{14} incorporated into DNA for single root tips. Roots were fixed and stained; the meristems thus revealed were cut off and smeared on copper planchets under a round cover slip in 45 per cent acetic acid. The planchet was placed on dry ice until the acetic acid froze; the cover slip was then pried off with no loss of cells. The smear was flushed cautiously with three 2 ml washes of 70 per cent alcohol to remove unincorporated thymine-C^{14} or thymidine-C^{14}, air-dried, and counted for radioactivity in an internal flow counter [26]. Further washing was found not to remove any radioactivity. The dry weight of a single smear was so small that no correction for self-absorption was necessary.

For determination of total counts, treated roots were quickly rinsed three times with 10 ml portions of water. The seed was removed and the whole root was placed in a drop of 45 per cent acetic acid (*V*/*V*) and squashed between two copper planchets. After freezing for one minute on dry ice, the planchets were pried apart, air dried and counted. Essentially the same procedure was followed for determination of total counts in the meristem.

Estimation of radioactivity in DNA, RNA and acid soluble fractions.—Roots treated with radiothymidine and radiothymine were washed by quickly dipping in three successive beakers of ice water. The last wash contained negligible radioactivity. Acid soluble and nucleic acid fraction of whole rootlets (1 cm) were separated by the cold and hot perchloric acid extraction procedure of Ogur and Rosen [23]. Holmes *et al.* [15] found satisfactory agreement between the perchloric and the Schmidt-Thannhauser methods for determination of RNA and DNA in roots of *Vicia faba*. After addition of $KHCO_3$ and centrifugation to remove the insoluble potassium perchlorate thus formed, aliquots were taken for measurement of radioactivity.

The use of radioautographs to determine the localization of thymidine-C¹⁴ in nuclei and chromosomes.—Root tips after treatment with radioactive thymidine for 18 hours were rinsed, hydrolyzed, stained, and squashed on glass slides. The cover slips were removed by freezing and the slides allowed to soak in water from three to five hours to wash out any thymidine not incorporated in the nucleus. Stripping film of Kodak Limited of London was used according to the technique described by Howard and Pelc [16] and Fitzgerald *et al.* [7]. The film was exposed for six days before developing.

RESULTS AND DISCUSSION

Incorporation of radioactivity in nucleic acids determined by the Ogur and Rosen procedure.—The distribution of radioactivity according to the Ogur and Rosen procedure [23] in roots treated with thymidine-C^{14} (0.42 μmoles per ml for 18 hours) was as follows: (1) 78.7 per cent of C^{14} was present in the acid-soluble fraction (unincorporated thymidine and small amount

present as nucleotide); (2) 1.3 per cent of C^{14} appeared in an overnight extraction with cold perchloric acid (RNA); (3) 20 per cent of C^{14} was in the hot perchloric acid extract (DNA). Since 94 per cent of the C^{14} in total nucleic acids resides in DNA, the cold overnight extraction can be omitted. The hot perchloric extract alone therefore gives a fair estimate of radioactivity in DNA.

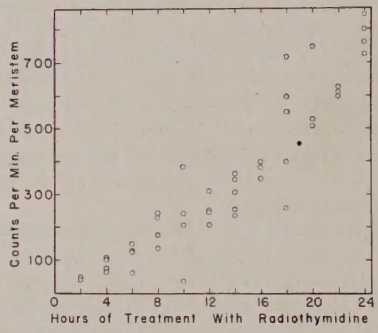

Fig. 1. Uptake of radioactivity into meristem DNA with increased exposure to thymidine-C^{14}. See methods (Schedule A and determination of radioactivity in smears) for description of procedure. Thymidine was present at a final concentration of 0.42 μmoles (1.26 μcuries) per ml of H_2O. ● average of 12 meristems referred to in text; ○ single meristems.

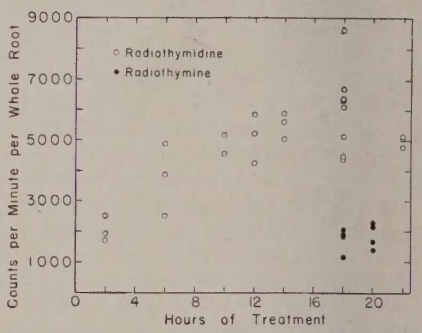

Fig. 2. Uptake of radioactivity in whole root with increased exposure to thymidine-C^{14} and thymine-C^{14}. *Total* counts in the root were determined by the planchet smear technique. Concentrations were the same as in Fig. 1.

The distribution of C^{14} in the DNA fraction and in the acid soluble fraction was compared in roots treated with thymine-C^{14} and thymidine-C^{14}. With radioactive thymine as a precursor only 125 counts per minute per root (entire rootlet) were found in the DNA fraction after an 18 hour treatment, whereas with radioactive thymidine 1380 counts per minute were incorporated. Furthermore, in radioactive thymine treated roots, the acid soluble fraction contained 1610 counts per minute per root compared with 4810 counts per minute for a radioactive thymidine treated root.

This not only indicates that thymidine is incorporated in DNA to a much greater extent than thymine (11:1) but that the total uptake of thymidine into the cell is greater than that of thymine (3:1). This was substantiated by the planchet count procedure to be discussed in detail in the following section.

Uptake of radioactivity by DNA of meristem determined by planchet smear technique.—Because thymidine is incorporated into DNA and not RNA, a convenient method for determining radioactivity in DNA per root tip was at hand. With the smear method described in an earlier section, 12 root

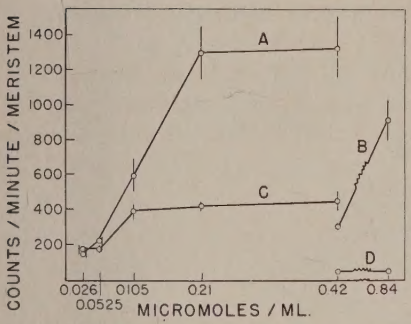

Fig. 3 (*left*). Planchet smear count determination of uptake of radioactivity by meristems at various concentrations of thymidine-C¹⁴ and thymine-C¹⁴. Total counts per minute in unstained meristems: *A*, thymidine-C¹⁴; *B*, thymine-C¹⁴. Counts per minute in DNA of stained meristems: *C*, thymidine-C¹⁴; *D*, thymine-C¹⁴. All values are an average of 5 or more meristems. The vertical lines indicate the standard error of the mean. All exposures were for 18 hours.

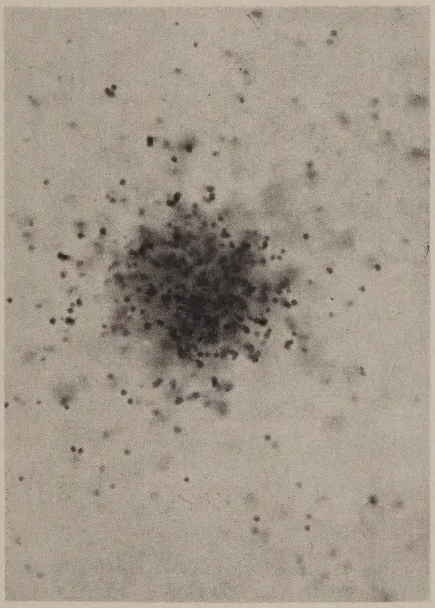

Fig. 4 (*right*). Radioautograph of thymidine-C¹⁴ labelled interphase nucleus of *Allium cepa* root meristem. About 4000 ×.

meristems treated with thymidine-C¹⁴ (18 hours, 0.42 μmoles per ml) assayed an average of 455 ± 10 counts per minute, while 12 roots treated similarly with thymine-C¹⁴ gave a value of 39 ± 0.97 counts per minute (± standard error of the mean).

Fig. 1 shows that with an increased exposure time to radiothymidine there is a linear increase of incorporation into DNA of meristems up to 20 hours. From 20 hours on there appears to be an uptrend. This linear increase would be expected of a cell population random with regard to the DNA synthesis period.

Rate of total uptake of thymidine-C¹⁴ and thymine-C¹⁴ into rootlets.—The rate at which radiothymidine and radiothymine enter the rootlet was determined by the smear count technique. Fig. 2 shows that thymidine-C¹⁴ reaches a maximum at about 10 hours; 80 per cent of the maximum is attained at about 6 hours. Despite the long treatment of 18 or 20 hours thymine-C¹⁴ reaches only about 40 per cent of the maximum thymidine value. This is in agreement with the data obtained by the perchloric procedure.

Uptake of radioactivity by meristems treated for 18 hours at various concentrations of thymidine-C¹⁴ and thymine-C¹⁴.—Thymidine at a concentration

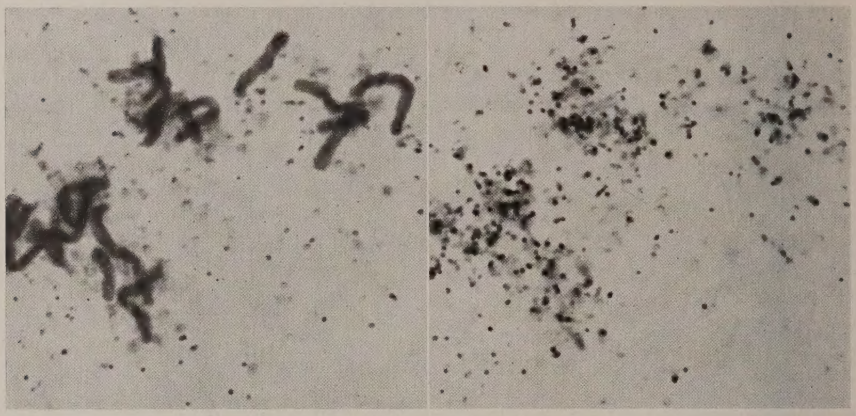

Fig. 5 (*left*). Feulgen stained metaphase chromosomes of thymidine-C^{14} treated meristem as seen through stripping film. About 3600 ×. Fig. 6 (*right*). Same field as in Fig. 5 showing radioautograph of treated chromosomes. About 3600 ×.

of 0.1 μmole per ml gave essentially the maximum incorporation into DNA of the meristem, Fig. 3; the maximum total uptake (counts in DNA plus all other counts) was reached at 0.2 μmoles per ml. Maximum uptake in the DNA represents about one-third of the total counts of the meristem. Of special interest is the fact that between 0.026–0.05 μmoles per ml the counts in the DNA equal the total counts.

Incorporation of thymine-C^{14} into meristematic DNA at concentrations of 0.42–0.84 μmoles per ml was only about 11 per cent of the maximum thymidine value. The total uptake of radiothymine at 0.42 μmoles per ml was 23 per cent of the radiothymidine uptake at the same concentration. These data are in substantial agreement with those obtained from the perchloric treatment of whole rootlets.

It is noteworthy that in order to obtain the same amount of total radioactivity in the meristem, 0.84 μmoles of thymine-C^{14} per ml was required in contrast to 0.1–0.2 μmoles of thymidine-C^{14} per ml.

Radioautographs with thymidine-C^{14}.—The use of thymidine-C^{14} for radioautographic studies of DNA synthesis was suggested by Reichard and Estborn [25]. In our studies radioautographs showed 80–90 per cent of the interphase nuclei were labelled after an 18 hour treatment with thymidine-C^{14}; all mitotic figures seen were labelled. Fig. 4 shows localization of radioactivity in an interphase nucleus; Fig. 5 shows localization in metaphase chromo-

somes. No labelling was encountered in stained but otherwise untreated root tips.

The use of thymidine-C^{14} for radioautographic studies of DNA synthesis eliminates many of the difficulties associated with P^{32} as described by Taylor and McMaster [29].

SUMMARY

1. By use of the perchloric acid procedure of Ogur and Rosen and by the planchet smear method it was shown that thymidine was utilized for DNA synthesis in meristematic cells of *Allium cepa* seedling root tips; thymine was virtually unused. This is in agreement with uptake studies in the rat, rabbit, chicken and *Tetrahymena*.

2. The total uptake (incorporated and unincorporated) of thymidine in meristems only or in whole roots is greater than that of thymine, (3:1). For thymidine the total uptake in the whole root reaches a maximum at about 10 hours.

3. The planchet smear was used to show the linear increase of thymidine incorporation in DNA of meristems for a period of over 20 hours.

4. The minimum concentration of thymidine which gave maximum incorporation into DNA was approximately 0.1–0.2 μmoles per ml.

5. Radioautographs of interphase nuclei and mitotic chromosomes were obtained with thymidine-C^{14} treated meristems.

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II. CHROMOSOME ABERRATIONS CAUSED BY THYMIDINE-2-C^{14} AND THYMINE-2-C^{14} IN THE ONION ROOT TIP[1]

Chromosome aberrations were studied in onion root tips treated with thymine 2-C^{14} deoxyriboside (thymidine) and with thymine-2-C^{14}. Roots treated with radioactive thymidine gave significantly higher aberration frequencies than those treated with equivalent amounts of radioactive thymine. A preliminary note of these findings has been reported [5]. The marked difference between thymidine and thymine uptake by meristematic cells has been discussed in Paper I [6]. In the present study an attempt was made to relate chromosome aberrations with the uptake of these radiocompounds.

MATERIALS AND METHODS

The general procedures for seed germination, treatment with isotope, fixing and staining are dealt with in Paper I [6]. Tips were smeared, mounted in 45 per cent acetic acid and cover slips sealed with sorbitol. Slides could be stored for some weeks at 4°C in a dessicator.

Cell scoring.—Anaphase and early telophase cells were scored for presence or absence of chromosome aberrations, the abnormalities consisting primarily of fragments and bridges. The aberrations were typical of X-irradiated material. Scoring was on the basis of per cent of aberrant cells rather than aberrations per cell. Only those cells were scored which were free of distortion and were not obscured by underlying or overlying portions of other cells. Only late anaphase and early telophase cells were scored because fragments were most easily detected at these stages. All root tips were coded so that the nature of the treatment was unknown to the scorer.

A count of cells with one or more micronuclei was made for all but a few of the root meristems studied. Meristems were found to contain 10,000 to 14,000 cells; 5–6 per cent of these cells were in mitosis and 2 per cent were anaphase and telophase

[1] This work was supported in part by grants from the American Cancer Society. The authors wish to express their thanks to Dr. Martin D. Kamen for his constant interest and advice.

cells. Approximately 50 scorable cells per root tip or about 0.4 per cent of the total cells met the above criteria for scoring. Four hundred and sixty-five tips were used in preliminary and final experiments.

RESULTS

Preliminary experiments indicated that a 19-hour exposure (a convenient overnight treatment period) to thymidine 2-C^{14} and thymine 2-C^{14} at level of 3.36 μmoles (10 μcuries) per ml produced chromosome aberrations.

This concentration is much higher than that necessary for maximum incorporation of thymidine into DNA [6] and since we were primarily interested in studying the effects of thymidine-C^{14} as part of the DNA structure, we reduced the concentration to 0.42 μmoles per ml or about one-eighth the original concentration.

Aberrations produced by thymidine-C^{14} and thymine-C^{14} at 0.42 μmoles (1.26 μcuries) per ml.—A 19-hour exposure to radiothymidine was more effective in causing chromosomal aberrations than a similar treatment with radiothymine (Table I). Controls (water, non-radioactive thymidine, non-radioactive thymine) showed much less chromosomal damage. A statistical analysis supported this conclusion. Another decisive difference between the effects of radioactive thymidine and radioactive thymine was apparent in the number of micronuclei produced.

Effects of duration of exposure to thymidine-C^{14} and thymine-C^{14} on frequencies of cells with chromosome aberrations.—The frequency of aberrant cells did not increase significantly with exposure to thymidine-C^{14} for periods up to 12 hours; after 12 hours, radiation damage increased markedly with exposure, whereas increasing exposure to thymine-C^{14} was not so effective

TABLE I

A comparison of frequencies of cells with chromosome aberrations induced by 19 hours' exposure to thymidine 2-C^{14} and thymine 2-C^{14}.

All components were present at a final concentration of 0.42 μmoles (1.26 μcuries) per ml of H_2O.

Treatment	Number of meristems	Cells scored	Aberrant cells (per cent)	Av. no. of cells per meristem with micronuclei
Radiothymidine	24	1,093	8.41	54.1
Radiothymine	24	1,084	2.12	11.3
Water	22	1,021	0.68	4.6
Non-radioactive thymidine	5	330	1.21	5.0
Non-radioactive thymine	5	264	1.13	7.4

TABLE II

Effects of duration of exposure to thymidine-C^{14} and thymine-C^{14} on frequencies of chromosomal aberrations.

Treatment (hours)	Number of meristems	Cells scored	Aberrant cells (per cent)	Av. no. of cells per meristem with micronuclei
Radiothymidine, 0.42 μmoles per ml (1.26 μcuries per ml)				
4	7	329	2.13	5.1
8	7	292	2.40	13.1
12	7	311	2.57	15.1
14	5	246	8.54	22.0
16	4	201	5.47	26.5
18	6	210	11.43	54.5
20	4	211	14.22	61.3
22	6	231	12.99	69.5
24	5	222	15.77	83.6
26	5	223	18.39	73.0
28	5	240	13.33	107.4
30	4	188	20.74	135.3
Radiothymine, 0.42 μmoles per ml (1.26 μcuries per ml)				
26	5	319	3.13	24.4
28	5	253	1.98	30.2
30	5	279	4.66	26.6

(Table II). The average number of cells per meristem with micronuclei increased slowly during the first 16 hours of treatment with radioactive thymidine, thereafter, the increase was much more rapid. Treatment with radioactive thymine for 30 hours did not produce such an increase.

It was clear from these experiments that radioactive thymidine caused chromosome aberrations which increased in frequency with increased exposure. This was not so for thymine. Thus there appeared to be a correlation between the aberration frequencies and the unique localization of radioactivity in the thymine of DNA, as shown by chemical studies and further substantiated by radioautographs in which the mitotic chromosomes were seen to be labelled. It was tempting to conclude that discharges in the nucleus were more important in causing chromosome aberrations than discharges originating in the cytoplasm. That this was not necessarily the case became apparent when it was found that cells of thymidine treated meristems contained more radioactivity not only in DNA but throughout the cytoplasm than those treated with thymine [6].

We therefore tried to design experiments which would tell us whether the chromosome breaks were caused chiefly by discharges in the DNA or by beta particles from extrachromosomal sources. Accordingly the following experimental approaches were made:

I. Roots were treated with thymidine-C^{14} of low and high specific activities while maintaining the same amount of radioactivity per ml in both baths. If aberrations were caused primarily by C^{14} in the DNA then these aberrations should be decreased by reducing the amount of C^{14} in the DNA.

II. Roots were treated with low concentrations of thymidine-C^{14} and high concentrations of thymine-C^{14} in order to obtain the same amount of total radioactivity in meristems of both groups. Thus cells containing C^{14}-labelled chromatin and extra chromosomal radiocarbon could be compared with cells in which essentially all of the radioactivity was located in the cytoplasm.

Approach I

The reduction of aberration frequency upon dilution of thymidine-C^{14} with non-radioactive thymidine.—If aberrations were caused primarily by C^{14} in the DNA, then these aberrations should be reduced by decreasing the specific activity of the thymidine-C^{14}. The treatment was so arranged that the beta flux around the rootlets was the same for those treated with radiothymidine of high and low specific activity. The resulting aberration frequencies appeared to be related to the specific activity (Table III). In roots treated with thymidine of low specific activity the radioactivity of the DNA was roughly one-third that of the DNA of roots treated with thymidine of high specific activity. The absolute uptake of thymidine into DNA was about the same in the presence of four times as much thymidine in the bath. The total amount

TABLE III

Reduction of aberration frequency upon dilution of thymidine-C^{14} with non-radioactive thymidine. Roots were treated for 18 hours with radiothymidine.

	Thymidine-C^{14}, 0.42 μmoles per ml (3 μcuries per μmole)	Thymidine-C^{14}, 1.68 μmoles per ml (0.75 μcuries per μmole)
No. of cells scored	484	387
Aberrant cells	39	13
Aberrant cells, per cent	8.05	3.36
Av. no. of cells per meristem with micronuclei	60	20
Counts per min. in DNA of meristem	447	145
Total counts per min. in meristem	1334	362

of thymidine entering the entire meristem was about the same despite the presence of increased thymidine. Therefore, although the counts in the DNA decreased as would be expected with decrease in specific activity, the drop in aberration frequency could not be attributed solely to fewer counts in the DNA since the total counts in the entire meristem also decreased.

Approach II

Aberration frequencies obtained with low concentrations of thymidine and high concentrations of thymine.—Thymine-C^{14} was used in these studies as a control in an effort to introduce a β source which would be within the meristem but not within the DNA. The realization of an adequate control was not completely fulfilled. Despite the fact that roots were treated with the same concentrations of thymine-C^{14} and thymidine-C^{14}, thymidine treated roots contained approximately 3.6 times the radioactivity of thymine-treated roots (Paper I [6]). Therefore not only was thymidine utilized for DNA synthesis but it also accumulated to a greater extent. In one experiment, paper chromatography indicated that a major portion of the unincorporated radioactivity could be attributed to unchanged thymidine and that the formation of a nucleotide incapable of freely diffusing in and out of the cell was probably not a factor in the accumulation.

In order to achieve the same β dosage within thymine and thymidine treated cells the effects of high concentrations of thymine were compared with low concentrations of thymidine (Table IV). Since non-radioactive thymine gave essentially the same effect as radiothymine at 0.84 μmoles per ml we can assume that there was no radiation effect. With thymidine at 0.105 μmoles per ml there was no statistical difference between the radioactive and non-radioactive forms. However, with thymidine-C^{14} at 0.21 μmoles per ml there was a definite radiation effect.

From Fig. 3 Paper I [6], it is evident that roots treated with radiothymidine at 0.16 μmoles per ml (an interpolated value) would contain the same amount of total radioactivity as roots treated with thymine at 0.84 μmoles per ml. The aberration frequency expected for this concentration of thymidine-C^{14} would be about 3.95 per cent compared to 0.43 per cent for thymine-C^{14} (these values are corrected for chemical effect. Table IV). These data support the hypothesis that disintegrations within the DNA are the chief cause of the chromosome aberrations observed.

Roots treated with radiothymidine at 0.1 μmole per ml contained very nearly as much radioactivity in the meristematic DNA as those treated with thymidine at 0.21 or 0.42 μmoles per ml (Fig. 3, Paper I [6]). Yet the per cent

TABLE IV

Aberration frequencies obtained with low concentrations of thymidine and high concentrations of thymine.

Conc. μmoles per ml		Radioactive	Non-radioactive	Radioactive minus non-radioactive	Statistical comparison of radiation and chemical effects (chi square)
		THYMIDINE*			
0.21	Number of roots	12	6		
	Aberrant cells	57	3		
	Cells scored	828	448		
	Per cent aberrant cells	6.88	0.67	6.21	16.8 ($p = 0.001$)
	Cells with micronuclei per meristem (average)	43	11		
0.105	Number of roots	11	6		
	Aberrant cells	21	6		
	Cells scored	645	383		
	Per cent aberrant cells	3.25	1.57	1.68	2.06 ($p = 0.15$)
	Cells with micronuclei per meristem (average)	18	9		
		THYMINE			
0.84	Number of roots	9	8		
	Aberrant cells	27	18		
	Cells scored	693	519		
	Per cent aberrant cells	3.90	3.47	0.43	0.06 ($p = 1.0$)
	Cells with micronuclei per meristem (average)	27	26		

* In addition to the data in the table, 7 roots treated with non-radioactive thymidine at 0.84 μmoles per ml gave a value of 2.51 per cent aberrant cells for 479 scorable cells.

of aberrations was low (Table IV) in comparison. This should not have been the case if the aberrations were due solely to radiothymidine in the DNA. It might well be argued that the aberrations are more closely correlated to the total radioactivity in the meristem than to the radioactivity in DNA alone.

DISCUSSION

It was found in these studies that increased exposure to radiothymidine caused increased chromosomal aberrations. The interpretation of this effect is necessarily complex. Aberration frequencies were determined by exa-

mination of anaphase-telophase cells. Whether or not an aberration could be detected in such a cell depended upon where that particular cell was in the mitotic cycle during treatment with radiothymidine. Thus the relation of treatment to such factors as (1) length of the mitotic cycle, (2) period of DNA synthesis in the mitotic cycle, (3) period of radiosensitivity and (4) time of cytological examination can be expected to influence the aberration frequency.

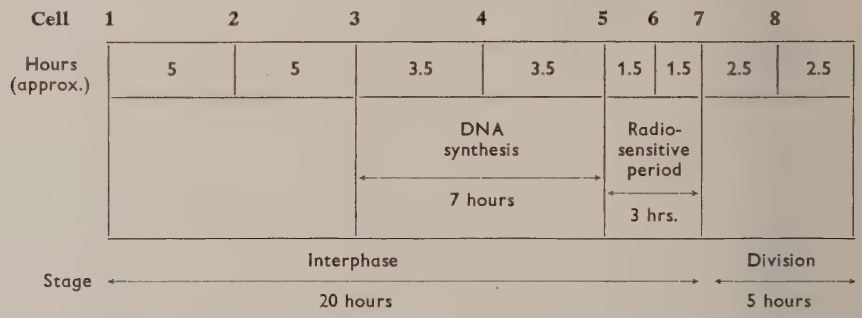

Fig. 1. Diagram of mitotic sequence in meristematic cells of onion seedlings.

Fig. 1 is a diagram of the mitotic sequence in the onion seedling root as suggested by studies in *Vicia* [2], roots of the onion bulb [7], bone marrow [4], as well as our own evidence from radioautographs [6].

On the basis of X-ray studies, the period of radiosensitivity is assumed to occur during the second half of interphase and is considered to be distinct from the period of DNA synthesis [8, 2, 1]. This period is not likely to be different for β radiation. Cells in the radiosensitive stage would be subjected to beta particles from unincorporated radiothymidine and from radiothymidine in DNA. Damage from transformation of C^{14} to N^{14} would be dependent upon amount of incorporation of radiothymidine into DNA and would not necessarily occur during the radiosensitive period. Radiomimetic chemicals are generally effective during the first half of interphase [8] but it has been recently shown by Kihlman [3] that in the onion the period of sensitivity to 8-ethoxy-caffeine, like X-rays, is in the second half of interphase.

In roots treated for 18 hours (Table II) cells scorable as late anaphase-early telophase probably had the following history: They were in early interphase when treatment began. (For example, Cell No. 2, Fig. 1). They had about 7 hours of labelling during DNA synthesis. They would have passed through a radio-sensitive period during the treatment.

In roots treated for 30 hours (Table II): Scorable cells were *daughters* of those probably in late interphase (for example, Cells 6 and 7, Fig. 1) at the beginning of treatment. These daughter cells arose from mothers which had passed through a radio-sensitive period during treatment with the possibility of sustaining hidden damage. The daughter cells then went through a full period of DNA synthesis with maximum labelling plus a subsequent radiosensitive period with thymidine-C^{14} present.

The above considerations emphasize the difficulties inherent in interpreting aberration frequency and micronuclei data obtained in cells which have incorporated radioactive materials while randomly distributed with regard to the mitotic cycle. But it seems reasonable that meristems treated for 30 hours sustained more damage than those treated for 18 hours because they contained more cells with maximum label and because the cells scored had not only passed through a radiosensitive period of their own but were derived from mothers that also had previously passed through a radiosensitive period in the presence of C^{14}. This points to the desirability of working with cells synchronized in mitosis.

CONCLUSIONS

We had hoped in these studies to demonstrate that the main source of chromosome aberrations in thymidine-C^{14} treated roots of the onion seedling is the C^{14} in the genetic material. We have not been able to do this decisively. Evidence for the C^{14} in DNA as the chief cause of aberration is the following: (1) Chromosome aberrations increased with increased exposure to radiothymidine; this was not the case with radiothymine. (2) When the concentration of thymine was raised to a point where radioactivity in the meristem was equal to that obtained with thymidine, chromosome aberration frequencies did not approximate those obtained with the latter. The main evidence against the C^{14} in DNA as the primary cause of aberrations was the finding that aberrations were closely correlated to total radioactivity in the meristem as a whole. We are aware that further experimentation is indicated.

A more definitive answer could be provided with the synthesis of thymine-C^{14} at least five times more active than the material available to us in the present studies; i.e., about 15 μcuries per μmole. With this intensely radioactive material the following experiments could be done. First, a comparison would be made of the aberrations caused by radiothymidine (3 μcuries per μmole) at 0.21 μmoles per ml and radiothymine (15 μcuries per μmole) at 0.42 μmoles per ml. This would give about maximum incorporation of

C^{14}-thymidine into DNA and the thymine would be of low enough concentration not to give a serious chemical effect. Most important, the total counts within the meristems would be the same in both cases. Second, thymidine-C^{14} with increased specific activity would permit a more critical study of post-treatment effects. Here rootlets would be grown in a bath of non-radioactive thymidine after treatment. In this situation, even though the unincorporated thymidine would be removed by equilibration, the specific activity of DNA might be high enough that radiation effects could be detected several cell generations later.

SUMMARY

1. Chromosome aberrations were studied in onion root tips treated with thymidine 2-C^{14} and thymine 2-C^{14}. Roots treated with radiothymidine gave significantly higher aberration frequencies than those treated with radiothymine. Chromosome aberrations increased with increasing exposure to radiothymidine; this was not the case with radiothymine.

2. Radiation effects of thymidine were studied because it is a precursor of DNA without diversion to any known cytoplasmic component including RNA. Thus a unique system is available for provoking intranuclear damage. Thymine enters the cell without becoming part of the DNA.

3. Experiments were designed to determine whether or not the main source of chromosome aberration in thymidine-C^{14} treated roots was the radiocarbon in the genetic material. Evidence for and against the hypothesis is presented. Additional approaches involving the use of more intensely radioactive thymine are outlined.

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THE FORM OF THE FREQUENCY DISTRIBUTION CURVE OF CELL AND NUCLEAR SIZES

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If the size of individual cells in a population or the size of their nuclei are measured and plotted against their frequency, then a whole series of differently shaped curves can be obtained depending on whether their size is expressed in a linear dimension, in surface area or in volume. There are some obvious statistical advantages in dealing with an unskewed normal distribution curve and therefore a number of workers have plotted their data in such a way that the most symmetrical distribution frequency possible was obtained. In these studies there arises the question of what is the most meaningful, logical method of plotting the data, and we will attempt to show here, that except under certain specific ideal conditions, it is unlikely that one can justify any particular method as being the most logical and that simplicity and straightforwardness, in the presentation of the data, therefore, continues to be the most desirable procedure.

Fitting curves in the graphical representation of biological phenomena is a notoriously difficult task, because of the scatter of the data, and measurements of cell size or nuclear size are no exceptions. In the first place, there is some error in the measurement itself, especially in cases where the part measured is irregular and inconsistent in shape. Another difficulty is that often not enough measurements are made, for accurate frequency distribution data require a large sample.

Let us take an example of cells that are approximately spherical—the cells of a slime mold after reaching equilibrium in a standard salt solution [2]. If the frequency is plotted against diameter of the cell the curve appears symmetrical (as in Fig. 3), and if the volume is calculated and plotted against frequency, then the curve is much skewed to the left (as in Fig. 1).[2] This is to be expected for if the diameter of linear dimension gives a symmetrical

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[2] It is possible, in a simple way, to test the symmetry by taking the relative cumulative frequency and plotting this on probability paper, for if the curve is a symmetrical normal distribution curve, then it should be a straight line when drawn graphically in this fashion. (See Figs. 5, 6.)

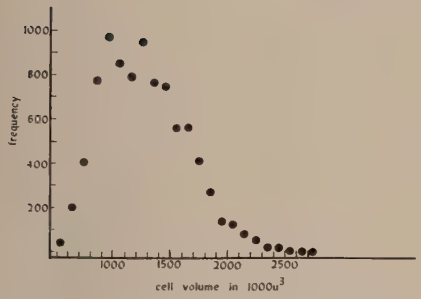
curve, then the volume would give a symmetrical curve only under very special circumstances. At the time of this work it seemed odd that it was specifically the linear diameter that would give a symmetrical curve and the rather fanciful suggestion was made that it might involve a critical ratio of surface to volume which would vary with the linear dimension.

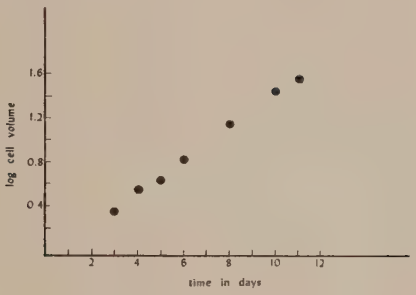
We were unaware then of the interesting work of Bucher (summarized in [3]) and his associates, who found that with nuclear sizes of tissue culture cells, it was possible to obtain a symmetrical curve with the logarithm of the volume of the nuclei. The logarithm of the volume tends to pull the distribution to the right (as does the diameter) and obliterate the skewness (compare Figs. 1, 3, 4). To determine in what cases the diameter or linear dimension and in what cases the logarithm of the volume gave the greater symmetry would be a laborious and tedious statistical problem and the result likely to be uncertain because of the scatter of the data.

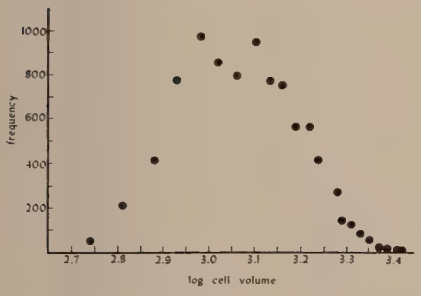
Bucher [3] holds out stoutly for the logarithm of the volume as the logical and rational measure. His reasons are not entirely clear but he states that "this method alone is correct from a biologic point of view, since in each case we are dealing with a doubling of the nuclear volume" (p. 74).

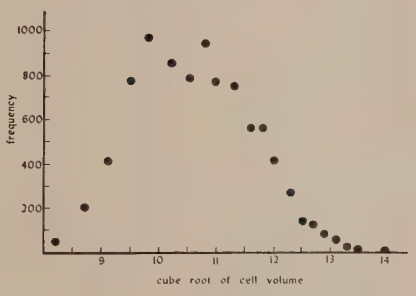
If by this Bucher means that the increase of the volume of a cell occurs logarithmically, then it is possible, provided the cells are all actively dividing, that the logarithm of the volume will give a symmetrical frequency distribution curve. But in the particular cells that Bucher studied, he has no data concerning their rate of increase in size between divisions, and judging from some other data of cells in tissue culture, it is quite conceivable that they do not increase logarithmically. See, for example, the data of Prescott [7] on cell size and the data of Fell and Hughes [4] on nuclear size, where in both cases there is a decrease in growth rate with time as the next division is approached, rather than a logarithmic increase.

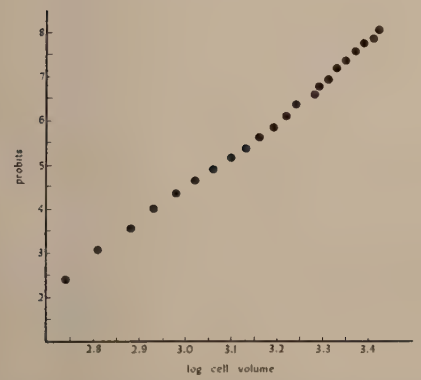
By good luck Gerassimoff [5, 6] has made a detailed study of spirogyra cells, which, being almost perfect cylinders, are easy to measure accurately, and he has measured a large sample. Because the intervals of the classes are most convenient with the series of cells with two nuclei [6] this series has been chosen for analysis. If the series is plotted, volume vs. frequency distribution, then, as one would have anticipated, the curve is much skewed to the left (Fig. 1). Now Gerassimoff [5] has measured the increase in volume of individual cells and found that their increase is logarithmic, that is, the logarithm of the volume plotted against time gives a straight line (Fig. 2). Therefore, one would in this case expect that if the logarithm of the volume were plotted against the frequency distribution, it would give a symmetrical

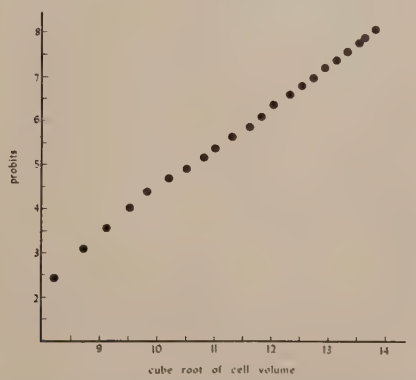












Size relations in spirogyra cells plotted in different fashions from the data of Geramissoff [5, 6]. *Fig. 1* (top, left): Frequency distribution vs. cell volume for 8851 cells. *Fig. 2* (top, right): The logarithm of the cell volumes vs. time in days, illustrating the logarithmic cell growth. Each point represents the average of 20 to 80 cells (redrawn from Adolph [1]). *Fig. 3* (center, left): Frequency distribution vs. the logarithm of cell volume. *Fig. 4* (center right): Frequency distribution vs. the cube root of cell volume. *Fig. 5* (bottom, left): The cumulative frequency (plotted in probits) vs. the logarithm of the cell volume. *Fig. 6* (bottom, right): The cumulative frequency (plotted in probits) vs. the cube root of the cell volume.

curve, and as one can see from Fig. 3, in a rough way, this is true. However, it is slightly disconcerting that if the cube root of the volume is plotted against the frequency distribution (Fig. 4) it also approaches a symmetrical curve, and if both of these are tested for linearity with the cumulation frequency plotted on probability paper, then both have a slight "s" shape, deviating somewhat from a clear, straight line (Figs. 5, 6).

The deviation from perfect linearity in the case of the logarithm of the volume may likely be caused by inaccuracy in the measurements or by the fact that there are other factors which affect skewness that we do not comprehend. The fact that the situation is incompletely analyzed is illustrated clearly in the case of the slime mold cells [2]. At some stages in the development of the slime mold there is active growth of all the cells undergoing periodic mitoses, but at other stages the cells are rigid and mature, such as the spore cells, yet in both cases the curve is skewed the same way and approximately to the same degree. Spore cells are obviously not increasing at a logarithmic rate; they are not increasing at all, yet they show the typical frequency distribution curve.

In an ideal example, for instance, a spherical cell, it would be necessary to obtain a reasonably detailed knowledge of the life history of individual cells before the distribution frequency for any measure of size could be described by a suitable model. It would include a knowledge of the mean time from division to division and the distribution function for this property; the size of each daughter cell immediately after division and the distribution function on size; the growth rate constant, if indeed it is constant, and *its* distribution function. Finally it would be important to know whether the number of divisions in a given time interval was proportional to the number of cells in the population, for it is not uncommon to observe divisions occurring in bursts, that is, as if the growth of different cells in the population were in phase.

The situation, therefore, even in its simplest form is a complex one that would require detailed information, often quite incidental to the main purpose of the investigation. There are many studies where it is useful to know the frequency distribution of cell or nuclear size, especially if two groups of cells are to be compared, or if certain cell types are to be followed in their development. From the considerations that have been set forth here we would conclude that unless a detailed analysis of the change of individual cells is made, the simplest and most straightforward presentation of the data is the most useful, and that in such instances any consideration of the meaning of the curve is not likely to be especially fruitful.

SUMMARY

If in a population of cells or nuclei, their size is plotted against their frequency, differently shaped curves can be obtained if the size is expressed in terms of a linear dimension, surface area, volume, or the logarithm of these functions. It was shown that only under very special conditions, when there is a wealth of details known about the growth characteristics of the individual cells or nuclei, can there be any "correct" or "logical" way of plotting the data. These conditions are, in fact, so stringent that they have never been met and therefore it is recommended simply that the data be presented in the clearest and most direct manner.

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PRESENCE OF ACCELERATOR AND RETARDING CLEAVAGE FACTORS IN AN EXTRACT OF OVARY IN SEA URCHINS[1, 2]

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The search for the possible existence of substances in protoplasm concerned with the regulation of cell division is a long-standing problem. The earlier studies had largely been concentrated in an effort to discover whether spermatozoa contain enzymatic material capable of inducing the development of mature ova [6, 21, 10, 22]. Allee and his collaborators demonstrated the presence of a readily extractable substance or substances from fertilized *Arbacia* ova which accelerate the rate of cleavage of such eggs to a statistically significant degree [1]. The active fraction was obtained from fertilized eggs that had passed through the third cleavage stage. The ova were ground with sand, centrifuged, and the supernatant was treated with acetone, alcohol, and 0.5*N* NaOH [1].

Considerable studies had indicated that extracts of various invertebrates retard development. Over thirty years ago Springer pointed out that *Arbacia punctulata* and *Asterias forbesii* contain substances which retard the development of the eggs of the same species [23]. Peebles reported that the eggs and larvae of sea urchin and starfish contain growth regulating substances [20]. The materials diffuse into the surrounding water during segmentation and in the later stages of larval development. The nature of these substances was not determined. In fact Peeble failed to remove any stimulating growth substances by dialysis. She was of the opinion that such substances may exist, but that their presence is masked by the stronger and more abundant growth-inhibiting factors [20]. In recent years Heilbrunn and his collaborators have demonstrated the presence of anti-mitotic substances in the acid sea water extracts of ovaries of starfish [7, 8]. The effect was a marked liquefying and anti-mitotic action on *Chaetoptherus* and *Arbacia* eggs. Most, though not all of the active principle, was found to be non-diffusible. These investigators have indicated that the potent substance may be a heparin-like compound [8].

[1] With the technical assistance of C. Gross and G. Menkin.

[2] Aided by grants from the U.S. Public Health Service and from the Dr. A. Wander, S.A., Berne, Switzerland.

Nucleic acid derivatives have frequently been pointed out to be concerned in growth responses. Almost twenty years ago, Loofbourow and his collaborators demonstrated that yeast cells injured by low intensity ultraviolet irradiation liberate a greater amount of proliferation-promoting substances than similar cells killed by exposure to higher intensity [5]. Spectroscopic analysis indicated an absorption range around 260 millimicra, characteristic of purines and pyrimidines of the nucleic acid complex [5]. These investigators also found that these active proliferating factors were diffusible through a dialyzing membrane, leaving an inactive residue behind. Ambellan recently demonstrated the precocious closure of the neural tubes in frog embryo treated with some mononucleotides, such as ATP, ADP, or AMP-3 [2]. Within the last year Skoog and his collaborators described a cell division factor derived from deoxyribonucleic acid (DNA) of some plants [18]. The name *Kinetin* was suggested for this slightly soluble substance, which is physiologically active, but only in the presence of auxins (indole acetic acid utilized) [18].

The present series of experiments, recently reported in brief [16, 17], indicate the existence of two opposed factors in the aqueous extract of the ovaries of the sea urchin, *Arbacia punctulata*. One of these factors diffusible in character and therefore very soluble in water, accelerates cleavage development of *Arbacia* ova. It appears to be a nucleotide. The other factor retards cleavage development. It seems to be a heavier molecule. Its exact nature has not as yet been determined. The presence of these two factors in the same tissue extract suggests that the rate of mitotic division may perhaps in part be regulated by these two biologically opposed factors.

MATERIALS AND METHODS

The ovaries of *Arbacia punctulata* were gently removed with forceps and homogenized in several milliliters of distilled water.[1] One ml of the whole extract was added to 9 ml of sea water in a Syracuse dish containing unfertilized *Arbacia* ova. The eggs were exposed to the whole extract for 20–30 minutes prior to fertilization. One ml of the extract was also added to a dish with unfertilized ova in order to determine if the extract *per se* had any effect in inducing development. A second control contained only ova in 10 ml of sea water. Fertilization following exposure of the ova was achieved by the addition of 6 drops of *Arbacia* sperm suspension. Each separate observation for the experimental and control dishes contained ova from the same sea urchin. This likewise held true for the sperms. Each reading represented counts on 50 ova, performed by two individuals. As soon as the first cleavage appeared, data began to be recorded on 50 ova to obtain the number of dividing and non-dividing ova. This

[1] The homogenizer employed was type 1288B, size "C": A. H. Thomas, Philadelphia.

procedure was repeated with little interruption between counts. One counting consisted of the ova scattered over several microscopic fields; and this counting was continued to the second and even further advanced stages of cleavage development. An experiment extended for an interval of about or over one hour. To avoid having one individual do all the controls or all the experimental counts, during the course of an experiment the counting dishes were often exchanged. By this checking device the personal error tended to be reduced. At the end of an experiment, the percentage of dividing and non-dividing ova was conveniently tabulated.

The whole extract was also centrifuged at about 4000 rpm in a Servall angle centrifuge for approximately 10 minutes. One ml of the supernatant was added to a Syracuse dish containing 9 ml of sea water with sea urchin ova. After exposure for 20–30 minutes the ova were fertilized. The procedure of counting the segmented eggs in both control and experimental dishes has been described above.

In a third series of observations 4 to 5 ml of the supernatant fraction derived from the whole aqueous extract was dialyzed against 20 ml of distilled water for several days in a refrigerator at about 10°C. The collected diffusate was found to be a clear yellowish fluid. Five tenths to 1 ml of this material was assayed on ova as already described. In some experiments studies on the thermostability of the diffusate were conducted by heating this fraction at 80°C to 100°C for intervals ranging from 30 minutes to about 1 hour. Furthermore, in a few experiments the diffusate was added to the ova several minutes after fertilization rather than following the reverse order. The indiffusible fraction following the termination of dialysis was likewise tested for its effectiveness. Some experiments were also performed to test the thermostability of this latter fraction.

In a final series of experiments, the residual or sedimented fraction of the original ovarian extract following centrifugalization, was assayed on developing eggs. In 2 experiments boiling of this residual fraction for 30 minutes was resorted to, prior to assay. Lengthening the period of exposure to that fraction was also observed in one experiment.

RESULTS

Some observations were made on the effect of the whole aqueous extract obtained from the ovaries of *Arbacia punctulata* (Table I). There resulted either no effect, a slight acceleration or a retarding effect on the cleavage development of the fertilized ova.

The supernatant fraction of the whole extract following relatively moderate speed centrifugalization (4000 rpm) tended to accelerate the incidence of cleavage to the first blastomeric division as well as to the immediate successive segmentations. The data are assembled in Table I. In 7 experiments in 44.1 minutes, an average of 32.6 per cent of experimental ova displayed an initial segmentation in comparison to an average of 15.4 per cent in controls. About 12 minutes later, an average of 22.3 per cent of the experimental ova were in the 3- or 4-cell stage, while the average figure for the controls was 8.6 per

TABLE I

Effect of the aqueous extract (whole extract and supernatant phase) derived from the ovaries of *Arbacia punctulata* on the cleavage of fertilized ova.

Exp. No.	Interval between fertilization and first cleavage (min.)	Per cent ova in 2-blastomeric stage (1st cleavage)		Interval between fertilization and 2nd cleavage or even further cleavage (min.)	Per cent ova in 3–4 blastomeric stage or even further advanced	
		Exp.	Control		Exp.	Control
1	45	26	14	47	18	0
2	47	18	2	53	22	14
3	48	48	26	72	14	14[a]
4	39	26	4	53	24	4
5	45	72	44	60	28	20
6	46	28	4	61	24	4
7	39	10	14	46	26	4
Average with supernatant phase of extract	44.1	32.6	15.4	56	22.3	8.6
8[a]	58	0	48	85	14	76
9[a]	41	22	6	43	16	10
	58	60	54	58	18	22
10	47	52	54	51	14	4
Average with whole extract	51	33.5	40.5	59.3	15.5	28

[a] This is whole reconstituted extract from the ovaries. It contains both the supernatant phase and the residual fraction following the mixture.

cent. At this point, it may also be mentioned that none of the various fractions obtained from the ovarian extract had *per se* any parthogenetic effect on the unfertilized eggs.

In examining the data it should be pointed out that the averages ought to be considered critically. The results of each separate experiment are of far greater importance and therefore should be primarily evaluated. Under the same environmental conditions of temperature, light, and with samples of ova from a given sea urchin, the rate of cleavage in the control is compared with that in the experimental. In the latter, either the whole extract, its supernatant part, the diffusate of this supernatant, or the sedimented fractions of the original ovarian extract has been added. It will readily be seen when the individual experiments are studied in the aggregated 52 observations that in

TABLE II

The presence of an accelerator cleavage or mitotic factor in the diffusate fraction of an aqueous extract of the ovaries of *Arbacia punctulata*.

Exp. No.	Interval between fertilization and appearance of cleavage (2-blastomeric stage) (min.)	Per cent of ova in 2-blastomeric stage		Interval between fertilization and appearance of 3–4 blastomeric stage (min.)	Per cent of ova in 3–4 blastomeric stage	
		Exp.	Control		Exp.	Control
1	43	30	2	43	6	0
2	38	26	0	44	20	0
3	39	34	14	39	16	2
4	52	62	4	86	56	12
5	60	42	0	85	24	10
6[a]	47	58	42	47	20	0
7[a]	40	34	0	44	10	0
8	43	72	54	43	4	8
9[a]	36	64	40	57	34	4
10[b]	99	4	56	109	48	0
11[a]	40	44	0	45	20	0
12	37	20	4	37	26	2
13	49	28	2	53	4	0
14	61	22	2	93	70	6
15[c]	56	34	4	85	32	4
16[c]	54	70	62	77	34	14
17[c]	45	42	40	65	34	12
18[c]	46	22	4	62	32	10
Average	49.2	39.3	20.3	61.9	27.2	4.7

[a] Diffusate heated from about 80°C to boiling for varying intervals ranging from 30 minutes to about 1 hour.

[b] Ova inadvertently exposed to sunlight for at least 2 hours on a very hot day. The question of damage to the ova arose.

[c] Diffusate added several minutes (2–10 minutes) after fertilization; in all others, ova exposed to diffusate prior to fertilization.

a given extract of sea urchin ovaries there exist both accelerator and retarding cleavage factors. This point of emphasizing the separate experiments is of significance in view of the considerable degree of variation in the onset of cleavage. This fact may render the reliability of depending only on the averages *per se* somewhat misleading.

The presence of an accelerator cleavage factor in the extract of ovarian tissue of Arbacia punctulata.—With the establishment that the supernatant of the whole extract tends to accelerate cleavage development, an endeavor was

made to obtain information on the nature of the factor concerned. As outlined above, the diffusibility at low temperature of the active principle was first demonstrated. The data of all the experiments are assembled in Table II. The accelerator factor seems to be recovered in its entirety in the clear yellowish diffusate. Therefore despite marked variations, 17 out of 18 experiments indicate the presence of the accelerator cleavage factor as a diffusible com-

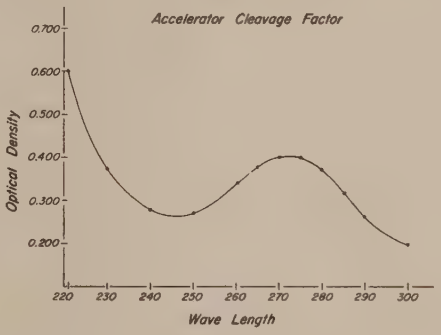

Fig. 1. Absorption spectral analysis with a DU spectrophotometer of the diffusate of an ovarian aqueous extract of *Arbacia punctulata*. This fraction contains the accelerator cleavage factor. Note the peak of absorption around 265 and 270 millimicra.

ponent. The average of all observations in 49.2 minutes reveals 39.3 per cent of ova treated with the diffusate in the first blastomeric division in comparison with 20.3 per cent in the controls. Subsequently, in an average of 61.9 minutes, 27.2 per cent of the experimental ova are in the 3–4 or more advanced cell stage, while the controls manifest at that level of development only 4.7 per cent ova. The material is relatively heat stable. Heating from 80°C to boiling temperature for thirty minutes to one hour does not appreciably alter the effectiveness of the accelerator cleavage factor (Table II). In 4 experiments the diffusate of the ovarian extract has been added several minutes following fertilization. Accelerated cleavage development appears to occur about as effectively as when the ova are exposed to the active principle prior to fertilization (Table II).

After having established the thermostability and diffusibility of the accelerator cleavage factor some measurements were conducted on the diffusate with a DU ultraviolet spectrophotometer. Some of these measurements were performed by Doctors J. S. Roth and M. Levy. The results obtained indicate an absorption peak around 265 to slightly over 270 millimicra (cf. Fig. 1). This finding suggests the presence of a nucleic acid derivative. In view of the diffusibility of the active component, its thermostability and its absorption spectrum, there is a strong possibility that the accelerator cleavage factor may be a nucleotide.

TABLE III

The effect on cleavage of ova of the indiffusible fraction of the dialyzed extract recovered from the ovaries of *Arbacia punctulata*.

Exp. No.	Interval between fertilization and first cleavage (min.)	Per cent ova in 2-blastomere stage (1st cleavage)		Interval between fertilization and appearance of 2nd cleavage or of further advanced stages (min.)	Per cent ova in 3–4 blastomeric stage (or even further advanced)	
		Exp.	Control		Exp.	Control
1	41	22	20	63	4	2
2	51	24	32	72	10	2
3	43	20	8	43	6	26
4[a]	41	6	14	49	20	12
5[b]	41	28	18	46	6	14
6[a]	46	0	4	51	4	0
7[b]	49	14	0	55	4	0
8[e]	40	28	32	45	12	4
9	47	2	14	54	14	60
10[d]	40	0	6	43	0	44
11[c]	57	46	46	86	22	62
Average	45.1	17.3	17.6	55.2	9.3	20.5

[a] Heated indiffusible factor for 30 minutes from 81°C down to 51°C.
[b] Same batch of ova in Exp. 5 as in Exp. 4 but unheated; same batch of ova in Exp. 7 as in Exp. 6 but unheated. Note the reverse effect.
[c] Boiled indiffusible material.
[d] 5 hours and 19 minutes' exposure to ova before fertilization instead of 28 minutes as in Exp. 9.
[e] In water bath at boiling temperature for about 1 hour.

The presence of a retarding factor in the ovarian tissue extract of Arbacia punctulata.—With the recovery of the accelerator cleavage factor in the diffusate of the ovarian extract, it became of interest to determine the biological potency of the indiffusible part of the extract following dialysis in the cold. The results of all observations are assembled in Table III. Out of 11 experiments, 8 revealed essentially no effect on cleavage development. At times there seemed to be either a slight accelerating or retarding effect in the onset of the 2-cell stage; but in such experiments examination of the next cleavages revealed usually a reversal of effects (cf. Table III). In one experiment (No. 10, Table III), with exposure of the ova prolonged to 5 hours and 19 minutes, evidence was obtained of a retardation in cleavage development. Otherwise retardation was encountered only in Exp. 9, Table III. Studies on the thermostability with the indiffusible fraction revealed no significant change

except perhaps a reversal of effects (cf. Exps. 4 and 6 and 5 and 7 respectively, Table III). Boiling for as long as one hour induced retardation only in the 3–4 blastomeric stage (Exp. 11, Table III). The average of 11 experiments with the indiffusible fraction of the extract elicited no change in the onset of the first division but apparently there resulted some retardation in the successive cleavages, the average in the experimental being 9.3 per cent in comparison with 20.5 per cent in the controls (Table III). In brief, the indiffusible fraction of the extract induced essentially no appreciable effect except, in some instances, moderate retardation in cleavage development subsequent to the initial segmentation. There seemed to be little doubt that this indiffusible part of the extract tended to yield inconstant results.

In contrast to the diffusible fraction, the indiffusible portion was found to be Biuret and Ninhydrin positive. Ammonium sulfate precipitation at one third saturation failed to yield an active fraction. As indicated in Table III and mentioned above, apparently some degree of thermostability seemed present, but boiling at times induced a reversibility of effects. Absorption spectrum measurements revealed with this indiffusible fraction a peak in the range of 265 to 270 millimicra. The findings were not wholly inconsistent with the possibility that a nucleoprotein might be involved. Nevertheless, the equivocal findings with this indiffusible fraction prompted the search for a different fraction to account for the retarding activity of the whole extract (cf. Table I).

The principal retarding factor was recovered in association with the residual or sedimented fraction following centrifugalization of the orginal extract. The results of 13 experiments are summarized in Table IV. With the exception of three experiments, the sedimented fraction of the extract following its centrifugalization, displayed appreciable retarding effects on cleavage development. Sometimes the retarding influence was encountered only at the first segmentation. At other times this was evident at the following stages of cell division (Table IV). Boiling of this fraction failed to alter appreciably its potency (Exps. 8 and 11). The addition of the active material four minutes following fertilization induced an effect similar to that produced by exposure to the retarding factor prior to fertilization (Exp. 13, Table IV). The average in the thirteen experiments indicated in 46.8 minutes, 10.3 per cent of the experimental ova in the first stage of cell division in comparison with 40.6 per cent in the controls. A similar effect was likewise reflected in the 3 or 4 blastomere stage; the experimental displaying an average of 19.1 per cent and the controls of 39.1 per cent. A question arises, namely the reason for the relatively high per cent of control ova in cleavage in contrast to the lower

TABLE IV

Retarding cleavage factor in the residual or sedimented fraction following centrifugalization of the whole ovarian extract.

Exp. No.	Interval between fertilization and 1st cleavage (min.)	Per cent ova in 2-blastomere stage (1st cleavage)		Interval between fertilization and 2nd cleavage or even further advanced stages (min.)	Per cent ova in 3–4 blastomere stage (or even further advanced stages)	
		Exp.	Control		Exp.	Control
1	43	10	22	52	8	2
2	39	0	24	71	44	70
3[a]	46	22	12	53	24	12
4	49	0	74	78	8	68
5	40	4	36	64	22	68
6	58	6	32	83	18	44
7	54	0	82	62	6	2
8[b]	41	18	76	65	30	56
9	49[d]	16	68	82	52	78
10	46	30	22	60	12	32
11[b]	48	24	10	61	22	34
12[c]	55	0	34	55	0	6
13	40[e]	4	36	44	2	36
Average	46.8	10.3	40.6	63.8	19.1	39.1

[a] The sedimented fraction was allowed to stand after further addition of distilled water. The heavier particles sank to the bottom of the container. The supernatant was collected and tried in this experiment. The possible presence of traces of the accelerator factor remains feasible.
[b] Boiled residual material for 30 minutes.
[c] Ova exposed to residual material for 3 hours and 12 minutes instead of 20–25 minutes as in Exp. 10 (same material and same batch of ova).
[d] 50 for control.
[e] Added 4 min. after fertilization.

average figure in other experiments (cf. Tables II and III). Examination, however, of Table I reveals a similar tendency in three of the four controls when the whole extract was employed (average 40.5 per cent, Table I). Scrutiny of Table II reveals that in 6 out of 18 experiments the controls likewise showed a high percentage of ova in the first blastomeric segmentation. Finally, as pointed out above, it is of greater significance to evaluate the results of each individual and well controlled experiment rather than to rely merely on the averages. The variations are considerable so that data from separate experiments as they accumulate in number, become of primary importance. In brief, there is but little doubt that the experimental results assembled in

Table IV lend definite support to the inference of the existence of a retarding cleavage factor in the aqueous extract, in turn derived from the ovarian tissue of *Arbacia punctulata*.

Finally, several observations were also made on development at longer intervals (e.g. after one day or thereabouts). Development to the plutei stage was occasionally observed with the accelerator cleavage factor; but this was found invariably absent with the retarding factor.

DISCUSSION

The present series of observations indicate that in the ovary extract of the sea urchin, *Arbacia punctulata*, there are two opposed factors apparently concerned in the regulatory mechanism of cell division. One of these factors, diffusible in nature, accelerates cleavage development of fertilized ova. The extent of acceleration varies; but in 18 experiments the incidence in the average onset of the first cellular division following fertilization appears to occur about twice as rapidly as in the controls. This effect seems even more pronounced in the second or third cleavage (Table II). The other factor is evidently a heavier molecule, for it readily sediments upon low speed centrifugalization of the extract. Biologically, it retards cleavage development. It is, in other words, anti-mitotic. The extent of retardation is quite marked, ranging in the first division, on the average, about 4 times as much as in the controls (Table IV). There is evidence by ultraviolet absorption studies as well as by other properties, that the accelerator factor may well be a nucleotide. Further studies now in progress, are required to characterize this more precisely, and to establish the nature of the retarding cleavage factor.

It is conceivable that the rate of mitotic division is the resultant of these two opposed factors. It remains to be determined whether such factors can likewise be recovered in other tissues as well as in other animal species.[1] The suggestion by absorption spectral analysis, diffusibility, as well as thermostability, that the accelerator factor may well be a nucleotide is interesting in view of the well known action of nucleic acid derivatives in regeneration [19]. Under some conditions derivatives of nucleic acid, as pointed out by Loofbourow, stimulate regeneration [11, 12]. Hörstadius, Lorch and Chargaff

[1] Studies, now in progress, indicate that an ovarian extract of *Arbacia punctulata* containing both the accelerator and the retarding cleavage factors can have similar accelerating and anti-mitotic effects on the cleavage development of the ova of a mollusc, namely the clam, *Spisula solidissima* (*Mactra*). These heterologous effects of an extract derived from an echinoderm on the cleavage development of ova from a species of a different phylum are doubtless of significance, and they will subsequently be reported *in extenso*.

have recently injected DNA, extracted from sea urchin sperms, into unfertilized eggs. They have found that upon mixing with the cytoplasm the stronger solution caused activation [9]. This was manifested by an elevation of the fertilization membrane, and in a few instances an abortive type of cleavage occurred, but which eventually resulted in cytolysis of the egg [9]. Berrian and Dornfield, on the other hand, have shown that all four nucleotides of PNA are anti-mitotic in rat ovaries [3]. Bullough and Green have also pointed out that *in vivo* adenyltriphosphate is inhibitory on mitosis [4]. Consequently, in view of the varied action of nucleotides [9], it is not surprising that one may be able to recover two opposed biological factors in the cell, in turn concerned in the regulation of cell division. It remains, however, to be established whether the retarding factor, as in the case of the accelerator cleavage factor, is likewise related to the nucleic acid derivatives. There is as yet no evidence for such an assumption.

Weiss has made interesting studies on the problem of specificity in growth control [24]. This investigator postulates, as a result of his experimental observations, the presence of intracellular specific catalysts (termed "templates") which under normal conditions remain confined in the cell. These specific substances seem to exert growth influences for differentiation of the homologous tissue from which they are derived. Each cell, according to Weiss, produces also inhibitory substances (termed "antitemplates"). These latter compounds are presumably constantly formed and released into the extracellular circulation. They contain also a specific affinity for the 'templates" of the cell from which they are derived and accordingly can inactivate them. In this way a decline in growth rate is favored with the increased liberation of these "antitemplates" into the extracellular medium [24]. This very interesting view of Weiss seems to be perhaps significant in reference to the present observations. It is conceivable that the accelerator cleavage factor may be associated with the "templates" of Weiss, while the retarding cleavage factor perhaps should be kept in mind in connection with the "antitemplates". It is hoped that future studies may throw further light on this point. Finally, in the last few years the writer has been able to demonstrate the presence of a growth-promoting or proliferative factor in the products of cellular injury recovered from an inflammatory exudate [15]. This factor is diffusible and appears to be perhaps a polypeptide [17]. Its polypeptide nature is indicated by preliminary chromatographic studies. There is no evidence that the factor is related to a nucleic acid or any of its derivatives [17]. The presence of this diffusible factor aids in explaining the mechanism of repair in inflammation [13, 17]. Furthermore, its growth effect is usually masked in an acute in-

flammation by the presence of an indiffusible toxic euglobulin termed by the writer, necrosin [14, 17]. These counteracting influences in areas of severe cellular injury in vertebrates as manifested in inflammation, should likewise be considered in connection with the presence of opposed biological factors in the regulation of the rate of cell division, as reported in the present study.

SUMMARY

1. An aqueous extract has been prepared by homogenizing the ovarian tissue of the sea urchin, *Arbacia punctulata*. This whole extract induces no significant effect on cleavage development of the fertilized sea urchin ova, or it may elicit either slight accelerating or retarding effects on the developing ova.

2. Centrifugalization of the extract yields on the part of the supernatant an accelerating effect on cleavage development; while the residual or sedimented portion induces a retarding cleavage effect on developing eggs.

3. Dialysis for several days of the supernatant fraction in the cold against distilled water favors the passage of an accelerating cleavage factor in the diffusate. This accelerator cleavage factor is also found to be thermostable, and ultraviolet spectral absorption studies indicate that one is dealing with a nucleic acid derivative. Diffusibility, thermostability, and absorption at 265 to around 270 millimicra suggest that the accelerator cleavage factor is a nucleotide.

4. The indiffusible fraction following dialysis yields inconstant results. There may be merely a slight inhibitory or retarding effect on the incidence of cleavage. There is the possibility from absorption and by simple tests for proteins that one may be dealing with a nucleoprotein in the indiffusible fraction.

5. The presence of an accelerator cleavage factor and of a retarding or antimitotic factor in the extract of the ovary in *Arbacia punctulata* suggests that the rate and therefore the regulation of cell division may perhaps be in part referable to a balance between these two basic and opposed factors.

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THE *IN VITRO* CULTIVATION OF THE PROSTATE GLAND OF THE ADULT MOUSE IN ALKALINE FLUID MEDIUM

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The watch glass method for organ culture has undergone many modifications and subsequent refinements since its introduction by Fell and Robison [2]. Recently renewed efforts in this field [7, 8, 1, 11, 12] gave impetus for a further investigation of useful techniques to be employed in the cultivation of organs of a highly secretory character.

This investigation was undertaken (1) to ascertain the further possibilities in the light of recent work in this field, of cultivating the ventral prostate gland of adult mice, and (2) to obtain some insight into the metabolic activity of this cultivated secretory organ as a basis for further physiological and metabolic studies.

After many attempts using various culture media and procedures [2, 37, 8] it was found that cultures of the ventral prostate gland of the adult mouse would grow and maintain themselves satisfactorily when explants of these organs were grown by the modified chamber technique [10, 11, 12], using the alkaline fluid medium of Pikowski [9], in a controlled gaseous atmosphere (5 per cent CO_2, 95 per cent O_2). With this method the connective tissue elements were suppressed, while a highly differentiated, richly growing, glandular tissue developed and could be maintained satisfactorily *in vitro* for 4 to 6 days.

TECHNIQUE

The ventral prostate glands were obtained from 40 adult CRW mice, aged 6–8 months. Halved ventral prostate glands were placed on a tantalum wire grid [11,12] and put in the lucite chamber designed by Trowell [11]. The entire apparatus including the gaseous atmosphere was maintained at 37°C. The gaseous atmosphere consisted of a mixture of 5 per cent CO_2 and 95 per cent O_2. The other lobe of the ventral prostate gland was used for control study. All controls after extirpation were immediately fixed.

The alkaline fluid medium of Pikowski [9] which consists of 40 per cent Earl's solution pH 9–9.3, 40 per cent horse serum, and 20 per cent chicken embryo extract was found to be the most satisfactory culture medium. Sufficient fluid medium (10

ml) is added to reach and just wet the flat surface of the grid. The cultures are planted on the wet lens paper covering the tantalum wire gauge. To prevent infection 250–300 units of pencillin G were added to the culture media [5]. With 15–20 organ explants in a chamber, it is necessary to refill the chamber with a fresh gaseous mixture once every day at the same time, and to change the culture medium every two days.

The explanted glands were fixed after the following periods of growth: 2 days, 4 days, 6 days, 8 days. For general topographical and cytological study both the explants and the controls were fixed by immersion in fluids of Bouin, Zenker-formal, Rossman, and 10 per cent neutral formalin. A number of staining methods were employed: Masson's trichrome; periodic-acid Schiff; Weigert's iron hematoxylin, fast-green, and safronin O; and hematoxylin–eosin. The tissues were embedded in paraffin and serially sectioned at 5 μ. Sections were cut at 2–3 μ for cytological study.

RESULTS

Both the epithelial and connective tissue elements of the explants (Figs. 2, 5, and 6) of the ventral prostate gland of the adult mouse cultured in the alkaline fluid medium of Pikowski [9] appear to be satisfactorily maintained for 4 to 6 days. In general, the results obtained with this method indicate that these explants resemble more closely morphologically and physiologically the normal ventral prostate glands of the adult mouse (Figs. 1, 2, 3, 4, 7 and 8) than has been heretofore reported [4, 5, 6].

In the cultured explants the secretory acini are lined for the most part by a single tall, cylindrical, folded secretory epithelium (Figs. 5, 6 and 7), which closely resembles the untreated normal control glands (Fig. 7). The acinar cells in the explants (4 to 6 days) tend to be taller and have clearer cytoplasm than the more granular acinar cells before explantation. The lining epithelium of the acinus displays a mild hyperplasia (Fig. 8) which

Fig. 1. Normal control prostate gland of adult mouse. Note the columnar, folded, secretory epithelium lining the acini and the delicate connective tissue stroma; 10 per cent neutral formalin; Weigert's iron hemotoxylin fast green and safranin O. × 150.

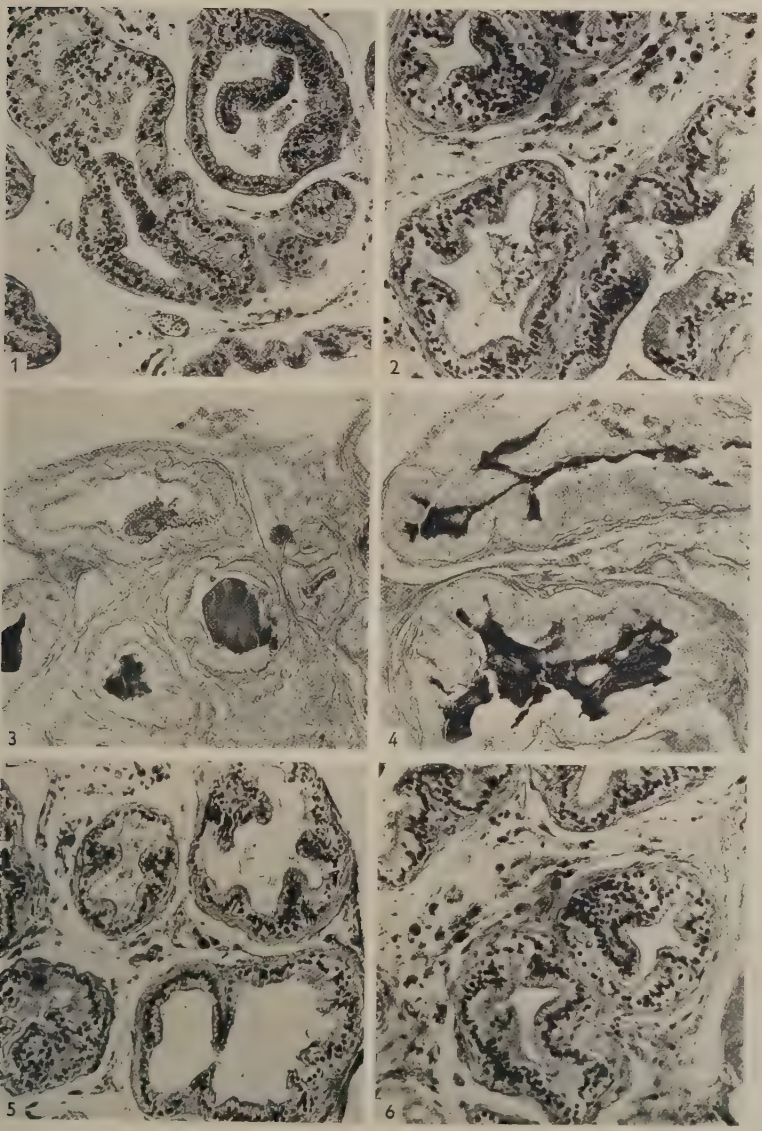
Fig. 2. Cultured 6-day explant. Both the epithelial and connective tissue elements appear to resemble very closely morphologically the normal control gland. Compare with figure 1; 10 per cent neutral formalin; Weigert's iron hematoxylin, fast green, safranin O. × 170.

Fig. 3. Normal control prostate gland of adult mouse. Periodic-acid Schiff positive material, probably polysaccharides, are present in the stroma, in the basement membrane, and in the secretion contained within the acinar lumen. Rossman's fluid; Periodic-acid Schiff. × 150.

Fig. 4. Cultured 4-day explant. The localization and characterization of the periodic-acid Schiff-positive material is the same as the control material. Rossman's fluid; Periodic-acid Schiff. × 170.

Fig. 5. Cultured 4-day explant. Both the epithelial and connective tissue elements appear to be satisfactorily maintained. 10 per cent neutral formalin; Weigert's iron hematoxylin, fast green, and safranin O. × 150.

Fig. 6. Cultured 6-day explant. A highly differentiated secretory epithelium is maintained, while the stromal elements are suppressed. 10 per cent neutral formalin; Weigert's iron hematoxylin, fast green, and safranin O. × 150.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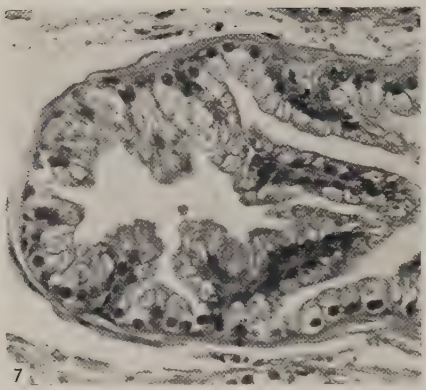
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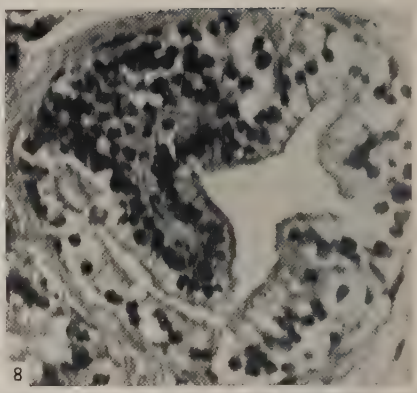
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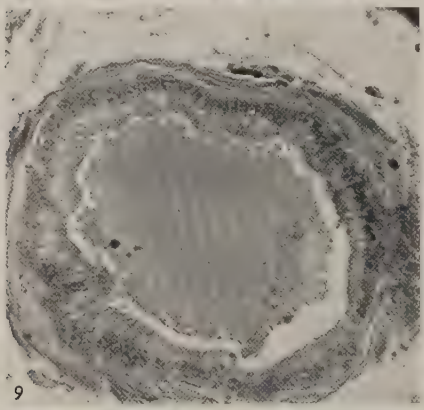
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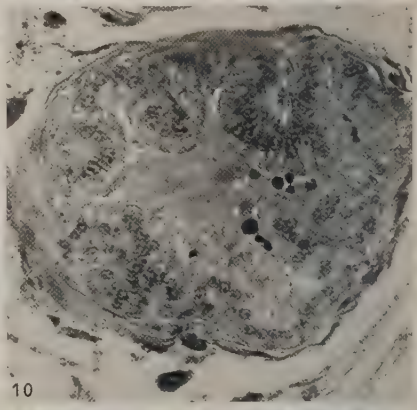
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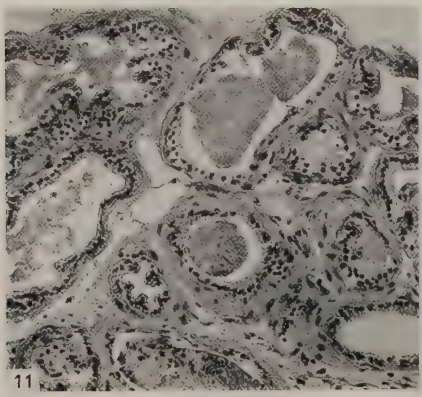
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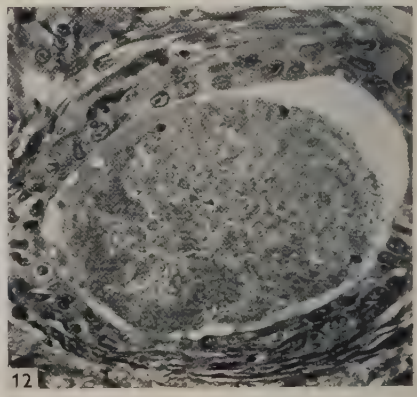
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is confined to one side of the acinar wall. In this area, there is an increase in the layers of reserve cells which are covered over by a single layer of secretory epithelium. Cell division is seen in the acinar epithelium but is not frequent.

The fibro-muscular connective tissue stroma of these explants (4 to 6 days) maintains its delicate appearance (Figs. 2, 5 and 6), and is similar to that found in the untreated controls. Periodic-acid Schiff positive material, probably polysaccharides, are present in the stroma in the basement membrane, and in the secretion contained with the lumen of the acini. Secretory antecedents are contained in the distal end of the secretory cell (Figs. 6 and 8) in the explanted glands (4 to 6 days). This secretion contained within the acini of the 4 to 6 days explants of the controls is homogeneous (Figs. 9 and 10), and is PAS positive (Figs. 3 and 4).

After six days of cultivation, the explants show several important changes. The highly differentiated epithelium, characteristic of the glands *in vivo* and in the 4 to 6 day cultured explants, disappears (Fig. 11). The lining epithelium becomes markedly flattened (Fig. 7) and cell boundaries are difficult to ascertain, so that it appears that the nuclei are contained in a ring of acinar cytoplasm. Cytoplasmic secretory antecedents disappear. The lumina are filled with plugs of coarse prostatic secretion, which is made up of shed disintegrating epithelial cells (Fig. 12). In addition, the connective tissue has undergone a marked hyperplasia (Fig. 11). The stromal elements, particularly the collagenous fibers have increased and are more extensive (Fig. 11). These explants resemble the prostate gland after treatment with estrone [5].

SUMMARY

Ventral prostate glands of the adult mouse were cultured and satisfactorily maintained for a period of 4 to 6 days in a modified chamber technique of Trowell [10, 11, 12] using the alkaline medium of Pikowski [9]. In com-

Fig. 7. Normal control prostate gland of adult mouse. The secretory cells have a granular cytoplasm and oval or round basal nuclei. 10 per cent neutral formalin; Weigert's iron hematoxylin, fast green, and safranin O. × 405.

Fig. 8. Cultured 6-day explant. The lining epithelium displays a mild hyperplasia, that is an increase in the number of reserve cells, which involves only a part of the acinar wall. 10 per cent neutral formalin; Weigert's iron hematoxylin, fast green, and safranin O. × 405.

Figs. 9 and 10. Cultured 4- and 6-day explants respectively. A highly differentiated secretory epithelium is maintained. Zenker-formal; Eosin, phloxin, methylene blue. × 405.

Fig. 11. Cultured 8-day explant. There is a marked hyperplasia of the connective tissue elements. Bouin's fluid; Masson's trichrome stain. × 150.

Fig. 12. Cultured 8-day explant. The acinar epithelium has become markedly flattened. Cell boundaries cannot be ascertained. Bouin's fluid; Masson's trichrome stain. × 150.

parison with the other media which were tried, the alkaline fluid medium of Pikowski [9] seems to enhance the maintenance of the secretory glandular epithelium, and to suppress the proliferation of the connective tissue elements.

In the explants cultured for 4 to 6 days, both the epithelial and connective tissue elements appear to resemble closely morphologically and physiologically their normal counter parts *in vivo*.

Upon cultivation longer than six days, the prostatic glands resemble histologically the gland of mice treated with estrone.

I gratefully acknowledge the skilled technical assistance of Miss Sheila Stahl. The author also wishes to express her thanks to Mr. Steve Montes for his aid in preparing the photographs.

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ISOLATION OF CHROMOPLASTS FROM CARROTS[1]

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A preliminary analysis of isolated chromoplasts from carrots had shown that their carotene content varies between 20 and 56 per cent [10]. It was suggested [10] that these variations depend on the age, variety and temperature during growth of the carrots, the same factors which, according to the work of other investigators, influence the carotene content of whole carrots. In the following pages it will be described that orange and red fractions of chromoplasts showing different carotene content can be isolated from the same batch of carrots. This leads to the conclusion that the different fractions represent different stages of development of the chromoplasts. The question is raised whether the content of the pigments is increased in a continuous, or in a discontinuous and regular manner during the development of the chromoplasts. Observations supporting the latter possibility will be described.

Concomitant with the accumulation of the pigments, the concentration of nitrogen (proteins), phospholipids and ribonucleic acids decreased [10]. Thus, the stroma of the chromoplasts is reduced while the pigments accumulate.

The red chromoplasts with high carotene content resemble carotene crystals although their strongly developed "border fibers" [12] immediately show that there is a difference. The orange spirals, rings, curved ribbons and filaments, etc., do not resemble carotene crystals. From the morphological characteristics of disintegrating chromoplasts [11, 12] and from the aspects of the precursors in living carrot cells [13], it was concluded that the crystalline shapes of the chromoplasts are determined mainly by the crystalline structure of the stroma. The effect of increasing concentrations of crystallised pigment on the shapes of the chromoplasts seems to consist only in making the curved forms more straight and more rigid.

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Principles of method of isolation

Before describing details of the procedure of isolation, the general principles will be discussed briefly.

In addition to orange and red chromoplasts the press juice of carrots contains many "spherosomes"[1] [5, 9] and an excess of other cytoplasmic granules of microscopic and submicroscopic dimensions. Most of this material cannot be separated from the chromoplasts by the usual method of differential centrifugation alone. A separation can be achieved, however, by centrifugation in a medium of high density. If the chromoplasts and adsorbed cytoplasmic granules are resuspended in 30 per cent sucrose solution and centrifuged at high speed ($15{,}000 \times g$) in an angle centrifuge, most of the orange chromoplasts are concentrated in two main fractions from which they can be recovered in relatively high purity: one fraction floating on the surface of the suspension and adhering to the wall toward the centrifugal side of the centrifuge tube *above* the surface of the suspension; the other main fraction adhering to the wall toward the centripetal side of the tube *below* the surface of the suspension.

The above mentioned "spherosomes" were separated, in a preliminary step of purification, by centrifugation in the same medium (30 per cent sucrose solution) at lower speed ($2000 \times g$). At this centrifugal force, the "spherosomes" rise to the top or adhere to the wall while the orange chromoplasts remain for the most part in suspension.

Although the chromoplasts are now highly concentrated, some granules are still present or may be formed anew by disintegration of some chromoplasts. "Differential resuspension", already applied much earlier [14], was used as a final step of purification. If the chromoplasts and remaining granules are spun down by high speed centrifugation from their suspension in distilled water, the main upper layer of the sediment consisting of pure chromoplasts goes into immediate resuspension by gentle shaking with distilled water whereas the lower layer containing chromoplasts and adsorbed granules does not resuspend easily. Both can thus be separated.

[1] The histological and histochemical properties of spherosomes have been described by Sorokin [9], Perner [5], and others. Perner [5] observed that the spherosomes form indophenol blue with the Nadi reagents and he concluded that they contain cytochrome oxidase. This opinion has been accepted by several investigators.

Practically pure spherosomes were isolated from the supernatant fluid of the red chromoplasts as described below and were tested for cytochrome oxidase by the indophenol blue [16] and quinonediimonium red [17] methods. Although Perner's observation could be confirmed that the spherosomes form indophenol blue, the same amount of pigment was developed in the controls containing NaN_3. Thus, spherosomes do not contain cytochrome oxidase. These experiments will be reported in another connection.

As was mentioned, an approximate density of the medium of 1.12 is required for the separation of the *orange* chromoplasts. In order to bring the *red* chromoplasts to the surface, the density of the original carrot juice (1.04 to 1.05) is sufficient. Thus, after high speed centrifugation of the original carrot juice, the red chromoplasts, contaminated with many spherosomes, are concentrated in the layer floating on the surface of the suspension and adhering to the wall above the surface. If this layer is taken up in distilled water and again centrifuged at high speed, the sediment consists of red chromoplasts in practically pure state while the spherosomes and lipid droplets separate on the surface and on the wall.

During the purification, the pH of the suspensions stayed around 6.5. Buffer solutions were avoided since added salts might increase the tendency toward agglutination of the contaminating submicroscopic particles.

Examples of isolation

Orange chromoplasts.—The carrots were washed, their surface scraped off, cooled in the refrigerator and grated through a machine with rotating knives. The brei was pressed through nylon cloth. Cooling was applied in all further manipulations.

600 ml of press juice were centrifuged for 30 minutes at 1100 rpm (150 × *g*) and the sediment was discarded. The chromoplasts in the supernatant fluid, together with most of the cytoplasmic granules, were spun down by centrifugation for 30 minutes at 11,000 rpm (20,000 × *g*) in the Servall model SS2 angle centrifuge, and the red chromoplasts were recovered from the layer floating on the surface according to the procedure described in a later paragraph. After decantation of the supernatant fluid the fatty material adhering to the walls of the tubes was rinsed out with distilled water without disturbing the sediments.

The sediments were shaken loose in whole pieces after addition of 30 per cent sucrose solution (adjusted to pH 8.0 with KOH) and were homogenized in 80 ml of the sucrose solution. The suspension was centrifuged for 15 minutes at 3800 rpm (2000 × *g*) in the Servall SS1 angle centrifuge in 50 ml tubes. The layer floating on top of the suspension and containing many spherosomes and some chromoplasts, was adsorbed as completely as possible on strips of filter paper and was discarded. The suspension was decanted and the centrifugation for 15 minutes at 3800 rpm was repeated once more and most of the remaining spherosomes removed as above.

The chromoplasts were then separated by centrifugation of the sucrose suspension for 45 minutes at 11,000 rpm (15,000 × *g*) in the angle centrifuge. The orange layers which floated on top of the suspension and adhered to the wall of the 50 ml tubes *above* the surface of the suspension were adsorbed on small, compressed pieces of dry absorbant cotton and were resuspended from the cotton in 15 ml of distilled water by pressing the cotton with a spatula, and were decanted. The chromoplasts which adhered to the wall of the tube *below* the surface were recovered in the following way. The suspension was decanted over the opposite (centrifugal) wall of the tube.

The heavy bottom sediment was completely removed by repeatedly adding small portions of distilled water, stirring up the sediment with a glass rod and pouring it out. The chromoplasts adhering to the wall were then resuspended in 15 ml of distilled water with the aid of a glass rod.

The chromoplast suspension in distilled water was centrifuged for 10 minutes at 1500 rpm (260 × *g*) and the sediment was discarded. The suspension was diluted with distilled water to 75 ml and the chromoplasts and most of the remaining granules were spun down by centrifugation for 30 minutes at 11,000 rpm (15,000 × *g*). The sediment, except the bottom part, was resuspended by repeatedly adding small volumes of distilled water, shaking gently, and decanting the resuspended chromoplasts until the remaining sediment failed to go into resuspension with avidity. If the microscopic examination showed that the suspension still contained methylene blue stainable granules [10], the differential sedimentation or resuspension was repeated once more.

The yield of completely purified "orange chromoplasts" was about 5 mgs from 600 ml of carrot juice. The yields were several times higher when contamination by some methylene blue stainable granules or fragments was tolerated.

The orange fractions always contained, besides straight rectangular sheets, many curved sheets and filaments: spirals, rings and other undulated forms. As mentioned before, the orange chromoplasts, especially those with low carotene content, show marked tendency toward fragmentation; those with higher carotene content much less.

Red chromoplasts.—After centrifugation of the carrot juice for 30 minutes at 20,000 × *g* as described in a previous paragraph, the red chromoplasts were recovered in the following way. Immediately after the centrifuge had come to a stop, the yellow layers which floated on the surface of the suspension in the 50 ml tubes and which contained the red chromoplasts together with an excess of spherosomes, were adsorbed on strips of filter paper (Whatman no. 1) and the filter paper was passed immediately into 140 ml of ice-cooled distilled water. The chromoplasts were resuspended by shaking and were decanted. The aqueous suspension was centrifuged for 30 minutes at 20,000 × *g* in the angle centrifuge. The fatty material which floated on the surface and adhered to the wall above the surface was removed with filter paper and absorbant cotton. The tube was then quickly turned upside down over a beaker and the fatty material adhering to the wall was immediately rinsed out without disturbing the red sediment. If microscopic examination of the resuspended chromoplasts showed them to contain still some spherosomes or fat droplets, the high speed centrifugation of the aqueous suspension was repeated once more.

A difficulty may arise from the possibility of the contaminating fat droplets dissolving some of the lipids of the chromoplasts. This applies especially to the red chromoplasts adhering to the wall of the tube above the surface of the suspension where most of the fat droplets are concentrated. The chromoplasts from this layer were not yet mentioned. They can be taken up with cotton and, after resuspension in distilled water, purified in the same way as described. The layer floating on the surface contained mostly spherosomes as contaminant and the latter did not seem to dissolve material from the chromoplasts. The yield of red chromoplasts was 5–10 mgs from 600 ml of carrot juice.

Analysis of the pigments

Total carotene.—In Table I, the carotene content of isolated chromoplast fractions is indicated. Most of the preparations are those already listed in Table I [10]. The majority of the fractions with carotene content around 19, 30 and 37 per cent were prepared as indicated above for the isolation of orange chromoplasts, those with carotene content around 56 per cent as described for the isolation of red chromoplasts. Some of the preparations, especially those with intermediate carotene content, were obtained when modifications of the procedure were tried out and before a routine procedure was adopted.. The purity of all preparations was tested by staining with methylene blue [10].

As may be seen from Table I, the isolated chromoplast fractions can be grouped into classes approaching constant carotene content and the values approach multiples of the same unit amount. These observations will be discussed below.

TABLE I

Classification of isolated chromoplast fractions into groups approaching constant carotene content.

Group	Per cent carotene per dry chromoplasts					Average	Calculated value ($n = 9.5$)
2*n*	18.3	18.3	18.9	20.0		18.9	19.0
3*n*	27.3	27.7	27.7	28.6	29.5		
	29.8	30.0	30.0	31.1	31.4		
	31.5	32.3	32.5			30.0	28.5
4*n*	36.7	36.8	37.0	37.4	38.8	37.3	38.0
5*n*	46.1	46.2	46.2	47.0	47.5		
	48.1	48.3				47.1	47.5
6*n*	55.0	55.6	56.2	57.2		56.0	57.0

α- and β-carotene.—An orienting experiment was made to test whether the different chromoplast fractions have the same or different concentrations of α-carotene and β-carotene. Three fractions, one red and two orange, were prepared from the same batch of carrots. The orange fraction II was obtained by continuing the centrifugation, after separation of fraction I, for another 30 minutes and recovering the newly formed wall sediment.

Petroleum ether extracts of the 3 fractions were poured onto 3 columns of $Ca(OH)_2$ [3]. After development with petroleum ether, the upper orange bands of β-carotene were separated from the lower yellow bands of α-carotene. The petroleum ether extracts of the eluted pigments were measured in the colorimeter.

The following results were obtained. In the red fraction, 65 per cent of β-carotene and 21 per cent of α-carotene, in both orange fractions 62 per cent of β-carotene and 20 per cent of α-carotene were recovered. Although the recovery of the pigments was not complete, the experiment seemed to indicate that there is no significant difference in the concentration of α-carotene and β-carotene in the different chromoplast fractions.

Colorless precursors of the pigments.—As may be seen from Table I [10], the pigments and phospholipids constitute the main portion of the ether soluble fraction of the chromoplasts but a considerable portion of this fraction remained unaccounted for. It may be expected that the colorless precursors of the pigments are major constituents of this fraction. Porter and Lincoln [6] have described colorless precursors of the carotinoid pigments such as phytofluene and phytoene and have discussed their genetic significance. Phytofluene has been found in carrots by Zechmeister and Sandoval [18], and phytoene by Rabourn, Quackenbush and Porter [7]. In the following orienting experiment the methods of these investigators were applied with slight modifications.

The petroleum ether extract of orange chromoplasts, after saponification with 5 per cent alcoholic NaOH, was poured on a column of $Ca(OH)_2$–Celite (3:1) and the adsorbed material was developed with petroleum ether until the bands of α-carotene and β-carotene were well separated. An intensely green fluorescing band appeared on the column in the intermediate zone between the two pigments. It was eluted, together with some α-carotene, and was rechromatographed on a column of MgO. The chromatogram was developed with petroleum ether containing 20 per cent ethyl ether until the green fluorescing zone had descended below the band of α-carotene. The petroleum ether extract of the eluted fluorescent substance showed the absorption maxima of phytofluene [18] at 348 mμ and 368 mμ when measured with the Beckman model DU spectrophotometer.

DISCUSSION

The question may be raised whether the carotene content is increasing in a continuous or in a discontinuous manner during the development of the chromoplasts and whether there exist regularities in the carotene content of these plastids. The data of Table I seem to suggest the latter possibility since the isolated fractions can be distributed into groups approaching constant carotene content and since the carotene values seem to approach multiples of the same unit amount. However, the following critical arguments have to be considered before definite conclusions are made. The isolated chromoplasts constitute only relatively small portions of all the chromoplasts of the carrots since many have been lost during the procedure of purification. It is possible that the experimental conditions of the procedure (density of the medium, times and speeds of centrifugation) tended to sort out fractions with similar carotene content. On the other hand, it is not certain whether the techniques applied would yield homogeneous fractions even if groups with

constant carotene content do exist. The statistical evaluation of a larger number of fractions than those shown in Table I should make it possible to decide the question.

There is another observation which seems to point to a regularity in the content of the pigments of the chromoplasts. As shown in Fig. 10 [12], and as may easily be seen on other, slightly damaged red chromoplasts, there are several layers superimposed in the chromoplasts of carrots. It can be recognised during microscopic examination of such chromoplasts that the thicker portions are more strongly colored than the thinner ones (see Fig. 10 [12]). It does not seem impossible that each new layer contributes a constant amount of carotene to the whole chromoplast. The increase of the thickness of the chromoplasts with increase of their pigment content can also be inferred from their decreasing tendency towards fragmentation as was mentioned before.

As early as 1885, Schimper [8] had noted that the chromoplasts of carrots appear in orange and in red shades. It was discussed [13] that these different shades may be caused by different concentrations of the pigments and also by the different degrees of dispersion of the pigments depending on the amount of accompanying proteins and lipids. Model solutions which showed similar absorption spectra as carrot juice were prepared by adding lecithin to colloidal carotene solutions of low degree of dispersion [15]. This model solution to the carrot juice showed, in addition to the absorption maximum in the green region of the spectrum, a stronger absorption in the blue region than did the colloidal carotene solution without addition of lecithin. In analogy to these experiments, it may be expected that the orange shades of the chromoplasts (strong absorption in the blue region) are influenced by the high concentration of phospholipids within the chromoplasts [10], whereas the red shades appear either in old chromoplasts with the regression of the stroma (phospholipids) and accumulation of the pigments, or in young chromoplasts where the higher concentration of accompanying proteins allows for closer association of the pigments with the proteins than with the phospholipids. The red, straight and thick chromoplasts with high carotene content, described in this paper, are examples of the first mentioned case, while the red, undulated and thin filaments and ribbons, to be seen in some carrot cells, are examples of the second mentioned case.

The crystalline shapes of the chromoplasts have found an interesting analogy in the crystalline structure of a central portion of young chloroplasts discovered by Heitz [1] and Leyon [4]. This will be discussed in a later paper in which certain properties of the chromoplasts of carrots and chloroplasts

from spinach leaves will be compared, and the results of the electron-microscopic investigation of the stroma of carrot chromoplasts, made in collaboration with Dr. E. A. Eckert, will be reported.

It may be mentioned that the same principles which were used for the isolation of the chromoplasts from carrots can be applied to the isolation of other plastids. Very pure chloroplasts from spinach leaves, for example, were isolated from the "wall sediment" and from the layer floating on the surface of a 45 to 50 per cent sucrose solution after high speed centrifugation of the partially purified spinach leaf homogenate (instead of the 30 to 35 per cent sucrose solution used in the isolation of carrot chromoplasts). A similar method has been described recently by Jagendorf [2]. By modification of the densities of the media, and of the times and speeds of centrifugation, it will thus be possible to purify chromoplasts and chloroplasts from other plants according to the same principles.

SUMMARY

Procedures were described for the isolation of orange and red fractions of chromoplasts from carrots. The fractions showed wide variations in their content of carotene and probably represent different stages of development of the chromoplasts.

Observations were reported which support the hypothesis that the carotene content of the chromoplasts of carrots varies in multiples of the same unit amount. The possible relation to the lamellar structure of the chromoplasts was discussed.

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A COMPARISON OF METHODS FOR ESTIMATING THE VIABLE COUNT OF A SUSPENSION OF TUMOUR CELLS

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The viable count of a suspension of tumour cells can be estimated in various ways. We term "viable" all cells which are capable of multiplication in a completely susceptible host. The methods commonly used include direct titration *in vivo* and various indirect *in vitro* tests, most of which measure some characteristic of the cells which is assumed to be correlated with viability. The only *in vitro* method which measures the ability to multiply is that described by Puck and Marcus [16] for use with HeLa cells. We have not traced any reports of experiments in which the validity of the indirect methods has been tested by comparing their estimates of the viable count with that given by the traditional direct method in which cells are inoculated into an animal host.

Direct titration *in vivo* can only give unequivocal results if any single cell of the test suspension invariably gives rise to a tumour. Hence, the ideal *in vivo* system contains a completely viable cell suspension and a completely susceptible host, the latter being defined as a host in which the inoculation of one viable cell will invariably give rise to a tumour. Such an ideal system can only be identified in practice by observing the manner in which hosts respond to inoculation and this depends partly on the method by which challenge doses are prepared. If doses are prepared by serial dilution of a concentrated cell suspension then, if the system is ideal, S, the proportion of hosts not producing a tumour following the inoculation of each host with a dose containing a mean total of d cells, is given by the first term of the Poisson series

$$S = e^{-d}.$$

At the ED 50 dose, $S = 0.5$ and hence d, the ED 50 contains a mean of 0.7

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cells. Hence, if an ED 50 of 0.7 is observed, one knows that the hosts are completely susceptible and that all the cells in the suspension are viable. If the ED 50 is more than 0.7 cells, then one cannot distinguish in practice between (1) a completely susceptible host challenged by an incompletely viable cell suspension, and (2) a resistant host challenged by a completely viable cell suspension. If it is assumed that the inoculated cells act independently so that there is a mean probability, p, per cell of producing a tumour, then in either (1) or (2), $S = e^{-pd}$, when the hosts do not differ in resistance.

The great practical disadvantage of *in vivo* methods for our work is that they only yield a result after a long period, ten weeks in our system. Hence one is forced to use some *in vitro* method in order to be able to perform quantitative experiments with a given cell suspension. The *in vitro* methods studied here are (1) the use of the stains trypan blue [14] and eosin [19] which are assumed to stain only dead cells; (2) incubation of the cells in a medium containing 2,3,5-triphenyltetrazolium chloride (T.T.C.; ref. [22]) which is reduced from a colourless oxidised state to an insoluble red pigment which is deposited intracellularly; and (3) exposure to trypsin which is said to digest only dead cells [13].

The results of *in vitro* and *in vivo* methods were compared using the ascitic form of the Krebs-2 carcinoma which has the advantage that only an insignificant proportion of cells are clumped.

Our observations show that, although an ideal system was not available, the *in vitro* methods may grossly overestimate the viable count and that the estimate given by one *in vitro* test is often incompatible with that given by another.

MATERIALS AND METHODS

Mice.—Two unrelated lines of randomly-mated mice were used: Male Albino mice supplied by H. Tuck & Sons, The Mousery, Rayleigh, Essex, and L.A.B. Grey mice bred at the London School of Hygiene and Tropical Medicine. They were fed on a cube diet (no. 41 or 86) and kept in groups of ten. At the time of challenge, their weights were between 18 and 25 g. Before each titration, they were allotted at random to different groups, disregarding sex, according to the tables given by Fisher and Yates [4].

Tumour.—The ascitic form of the Krebs-2 carcinoma was obtained from the Chester Beatty Institute in 1953 and maintained by serial passage in the peritoneal cavity of the Albino mice.

Preparation of cell suspensions.—In each experiment, one mouse, which had developed gross ascites following the inoculation of about 10^7 cells nine days before, was killed by decapitation. Ascitic fluid was withdrawn and collected in a sterile centrifuge tube. Each sample was washed in Earle's saline [3] by 3–4 centrifugations

at 220 *g* and the cells finally suspended in this medium for use in the tests. The total count was then about 10^7 cells per ml.

Total and diffential counts.—These were done in a Neubauer haemocytometer.

In vitro methods.—(*a*) Trypan blue: 0.1 ml of cell suspension was added to 1 ml of a 0.5 per cent solution of trypan blue (Gurr; vital) in phosphate buffered saline, pH 7.4 (P.B.S., ref. [2]). The differential count was made after 1–2 hours incubation at 37°C. Aged solutions seemed to give irregular results and all our observations have been made with solutions that were less than two weeks old.

(*b*) Eosin: 0.1 ml of cell suspension was added to 1 ml of a 0.05 per cent solution of eosin (Gurr; W.S. Yellowish) in P.B.S. The differential count was made after two minutes incubation at room temperature. Cells scored as dead were stained intensely in this time; other cells became stained light pink if the suspension was incubated for more than two minutes and these were scored as viable.

(*c*) T.T.C.: 0.1 ml of 1 per cent solution in P.B.S. was added to 2.5 ml of a medium composed of Earle's saline, 64.6 per cent; mouse ascitic fluid, 35.0 per cent; chick embryo extract, 0.4 per cent; penicillin 10,000 i.u. per cent and streptomycin, 20 mg per cent [10]. To this volume of medium was added 0.25 ml of cell suspension. Control experiments showed that the shortest period of incubation needed at 37°C for the count to reach a maximum was $5\frac{1}{2}$ hours. All suspensions were incubated in darkness. Reduced T.T.C. appeared as intensely red, discrete granules in the cytoplasm of cells scored as viable.

(*d*) Trypsin: 0.5 ml cell suspension was added to 5 ml of a 0.5 per cent solution of trypsin (Difco, 1:250) in P.B.S. and was incubated with P.B.S. alone. After trypsinisation, "ghosts" of digested cells could be seen.

Statistical methods.—The LD 50 was usually computed by Thompson's method [23] and the value of p and its standard error by the method given by Peto [15]. Moran's test [12] was used to detect incompatibility between the observations and the predicted dose-response curve, $S = e^{-pd}$.

Performance of titrations in vivo.—All mice were challenged with 0.1 ml suspension administered by intraperitoneal injection.

The Albino mice developed gross abdominal distension at varying times after challenge, the survival period being markedly influenced by the size of the dose, while Grey mice survived a given dose for a slightly shorter time but died suddenly without becoming distended. At *post mortem*, the subcutaneous tissues of the Grey mice were blanched and the peritoneal cavity was filled with blood in which tumour cells could be seen on microscopical examination. It was difficult to compare the survival times of the two lines as the Grey mice, being less variable in their response than the Albino mice as can be seen from the dose-response curves, died within a short time of each other whereas the survival times of individual Albino mice challenged by a given dose differed considerably.

RESULTS

Comparison of the in vitro methods.—This was done with a freshly harvested cell suspension and various suspensions which had been stored at 4°C for $3\frac{1}{2}$–11 days. Each suspension was examined before and after trypsinisation.

If trypsin digests only dead cells, as postulated, then all cells surviving trypsinisation should be scored as viable by the other methods. A fresh suspension which had been heated for 5 minutes in a boiling water bath was also examined.

Table I gives the results of some typical experiments. The percentage of viable cells and the ratio, number of cells scored as viable/total number of cells counted of each suspension, are given. With the freshly harvested suspension the estimated percentage viability was about the same by all the methods (92 per cent). It can be seen from the second and sixth columns that trypsinisation never destroyed all cells subsequently scored as dead by the other methods. Heated cells were scored as dead by all the methods. When suspensions stored at 4°C were examined, the estimates given by different methods differed more widely the longer the cells had been stored. After 11 days, 30 per cent of cells were scored as viable using eosin or trypan blue, 14 per cent by the trypsin method and less than 0.1 per cent were capable of reducing T.T.C.

Table I

The estimates of the percentage of viable cells given by four *in vitro* methods. The table gives the estimated percentage viability by each method and the ratio no. "viable" cells/total no. cells counted.

Method	Freshly harvested suspension			Suspension stored at 4°C for			
					5 ½ days		
	Before trypsinisation	After trypsinisation	After heating to 100 °C	3 ½ days	Before trypsinisation	After trypsinisation	11 days
Trypsin	94.0 % (408/434)	(100 %) —	< 0.1 % (0/1000)	59.7 % (700/1173)	46.4 % (474/1022)	(100 %) —	14.0 % (177/1265)
T.T.C.	92.5 % (492/532)	96.4 % (379/393)	< 0.1 % (0/1000)	81.6 % (1004/1231)	69.4 % (597/860)	92.8 % (360/388)	< 0.1 % (0/1000)
Trypan blue	88.4 % (434/491)	92.7 % (435/469)	< 0.1 % (0/1000)	75.6 % (910/1204)	64.1 % (430/669)	94.4 % (437/463)	29.9 % (368/1229)
Eosin	92.0 % (447/486)	97.4 % (414/425)	< 0.1 % (0/1000)	78.5 % (977/1245)	74.8 % (854/1142)	98.2 % (496/505)	30.5 % (368/1206)

Estimation of the viable count of freshly harvested cell suspensions by titrations in vivo.—Two unrelated lines of mice, referred to as Grey and as Albino, were available in large numbers. A preliminary titration using ten-fold

TABLE II

The responses of two unrelated lines of mice to challenge by a freshly harvested cell suspension.

GREY MICE								
$\log_{10} d$	3.23	2.23	1.23	0.23	Ī.23			
$1-S$	6/6	6/6	5/6	4/6	0/6			
	LD 50 = 2.15 cells							
$\log_{10} d$	1.3	1.0	0.7	0.4	0.1	Ī.8	Ī.5	Ī.2
$1-S$	15/17	10/17	9/17	5/17	5/17	0/15	2/17	0/17
	$p = 0.138$ (95 per cent fiducial limits: 0.097–0.179)							
	LD 50 = 5.07 cells							
ALBINO MICE								
$\log_{10} d$	6.23	5.23	4.23	3.23	2.23	1.23	0.23	Ī.23
$1-S$	6/6	6/6	4/6	4/6	4/6	4/6	1/6	0/6
	LD 50 = 29.5 cells							
$\log_{10} d$	2.2	1.9	1.6	1.3	1.0	0.7	0.4	0.1
$1-S$	9/20	12/20	8/16	7/18	4/17	3/18	0.20	0/20
	LD 50 = about 100 cells (by inspection)							

$\log_{10} d$ = logarithm $_{10}$ of mean total number of cells per dose.
$1-S$ = no. mice dying/total no. challenged.

dilutions of a freshly-harvested suspension was performed in each line with the results given in Table II.

The Grey mice were more susceptible than the Albinos and the slope of the dose-response curve appeared to be steeper. The titrations were then repeated using two-fold dilutions and larger numbers of mice with results that confirmed those of the first titrations. A single cell suspension was used in each pair of titrations so that the responses of the Grey and Albino mice could be compared. The dose-response of the Grey mice was compatible with the predicted curve, $S = e^{-pd}$, ($\chi^2 = 9.35$; 7 d.f.; $0.3 > P > 0.2$), where p is the mean probability per cell of multiplying in the given host. The observed value of p was 0.138 giving an LD 50 of 5.07 cells. Hence, the estimated percentage viability by this method was equal to or more than 13.8 per cent; the eosin method gave an estimate of 95 per cent on this suspension. The dose-response curve of the Albino mice was incompatible with the predicted curve ($\chi^2 = 30.3$; 7 d.f.; $P < 0.001$) indicating that individual mice differed significantly in susceptibility. The LD 50 was about 100 cells by inspection.

A comparison of in vitro methods and titrations in vivo using fresh and stored cell suspensions.—A washed cell suspension was divided into two portions. The first was inoculated into mice at once and its viable count estimated by the *in vitro* methods. The second portion was stored at 4°C for 11 days and then treated in the same way. Only Albino mice were used in this experiment as the development of ascites made it easier to recognize deaths due to nonspecific causes. The results are shown in Table III.

TABLE III

The effect of storage at 4°C for 11 days on the estimates of percentage viability by different methods.

LD 50 fresh cells/LD 50 stored cells = $372/10^6$. Hence 0.1 per cent of stored cells were viable.

	Trypsin	Trypan blue	Eosin	T.T.C.
Fresh suspension	94.9 % (811/854)	95.0 % (781/822)	96.8 % (962/993)	Not done
Suspension stored at 4°C for 11 days	2.4 % (23/942)	< 0.17 % (0/591)	17 % (159/928)	< 0.12 % (0/843)

The table gives the estimated percentage viability by each method and the ratio: no. "viable" cells/total no. cells scored.

		Details of titrations in Albino mice							
Fresh suspension	$\log_{10} d$	6	5	4	3	2	1	0	Ī
	$1 - S$	5/5	5/5	2/5	4/5	2/5	1/5	0/5	0/5
		LD 50 = 372 cells.							
Suspension stored at 4°C for 11 days	$\log_{10} d$	6	5	4	3	2	1	0	Ī
	$1 - S$	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
		LD 50 = 10^6 cells							

$\log_{10} d$ = logarithm $_{10}$ of mean total number of cells per dose.
$1 - S$ = no. of mice dying/total no. challenged.

The dose-response curve of mice injected with the fresh suspension was again incompatible with that predicted from the Poisson series. The observed LD 50 was 372 cells. The percentage viability was estimated at about 95 per cent by the trypsin, trypan blue and eosin methods. After 11 days storage at 4°C, even a dose of 10^6 cells (= 10^3–10^4 LD 50 doses of fresh cells) failed to produce a tumour in even one out of the five mice showing that the pro-

portion of viable cells was now less than 0.1 per cent of its initial value. The results of the *in vitro* methods now differed greatly. The trypan blue and eosin methods gave estimates of less than 0.17 per cent and 17 per cent viability respectively and for the first time, their estimates differed markedly. The trypsin method gave an estimate of 2.4 per cent. The method using TTC gave an estimate of less than 0.12 per cent.

DISCUSSION

The *in vitro* methods used in our experiments gave reasonably compatible results with fresh and with boiled cells but differed greatly when applied to suspensions stored at 4°C for varying times. The estimated percentage viability of a suspension stored for eleven days at 4°C was in one experiment, about 30 per cent by the eosin and trypan blue methods, 0 per cent by the T.T.C. method and 14 per cent by the trypsin method. In another experiment with a similar suspension, these methods gave estimates of 17, less than 0.17, less than 0.12 and 2.4 per cent respectively when titrations *in vivo* showed that less than 0.1 per cent were viable. Our experiments show therefore that the *in vitro* methods can give very inaccurate estimates of the viable count and their estimates do not bear a constant relationship to each other.

It is reasonable to suppose that each method measures a different property of the cell. For example, the reduction of T.T.C. presumably depends on the functioning of intracellular dehydrogenases [20] while staining by trypan blue and eosin depend on the integrity of the cell surface. Our results show that these properties do not alter at the same rate in cells stored at 4°C. One would always expect these methods to give incompatible results with cells exposed to harmful agents which acted either specifically or at a rate slow enough for interference with specific functions to be detectable before gross cellular changes followed. On the other hand, the methods should give compatible results with suspensions which have been exposed to a grossly unfavourable environment, such as a bath of boiling water, which would derange all cell functions very rapidly. Apart from this cause of discrepancy, it is known that the *in vitro* methods can differ when applied to certain types of cell which are clearly capable of multiplication in tissue culture; for example, monkey kidney cells which are able to multiply *in vitro* are all stained by eosin [18] while chick fibroblasts growing under similar conditions fail to reduce T.T.C. [21]. These experiments could only have given unequivocal results if an ideal system had been available as a control, that is, a system containing a host and a cell suspension in which the inoculation

of one cell would always give rise to a tumour. Puck and Marcus [16] have shown that an ideal tissue culture system is available for use with HeLa cells. It was stated in the introductory paragraphs of this paper that when inoculations are made from serial dilutions, the dose-response curve of an ideal system would be given by $S = e^{-d}$ and that the ED 50 is 0.7 cells. The LD 50 using the Grey mice was 5 cells and the dose-response curve was compatible with $S = e^{-p\,d}$ with $p = 0.14$. This system was therefore not ideal.

It seems unlikely that an ideal system using a living host has ever been described in the literature. Many experiments have been reported in which mice were inoculated with doses prepared by serial dilution. The smallest reported ED 50 doses we have traced are 14 [7] (dosage was measured in terms of cells not stained by eosin); 34, 18 and 9 [8, 9] (dosage measured as before); 32 [1]; 13 [17] (this refers to "viable" cells). There have also been reported several experiments in which known numbers of cells have been isolated and injected by the use of a micromanipulator. To establish with a conventional degree of certainty that a given system was ideal, at least 95 per cent of single cell inoculations would have to result in the growth of a tumour. We have not found reports of any experiments in which this result was obtained [5, 6, 11].

SUMMARY

The principles underlying the estimation of the viable proportion of cells in a suspension are discussed, viability being defined as the ability to multiply in a completely susceptible host. All estimates give equivocal results unless an ideal control system is available in which the inoculation of one cell always produces a tumour. The dose-response curves of such a system are defined.

The results of four indirect *in vitro* methods of estimating the percentage viability of a cell suspension have been compared with the results of titrations in mice. The most reliable indirect index of viability was the ability to reduce triphenyltetrazolium chloride. All four *in vitro* methods gave similar results with a recently harvested cell suspension but three of them grossly overestimated the proportion of viable cells in a suspension which had been stored at 4°C for 11 days.

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THE INFLUENCE OF THE JELLY COAT *IN SITU* AND IN SOLUTION ON CROSS FERTILIZATION IN SEA URCHINS

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The importance to fertilization of the jelly layer surrounding the sea urchin egg has been discussed intensely [6, 13, 14, 21, 30, 37]. In his "fertilizin theory" Lillie [20, 21] assumed that the jelly layer is saturated with "fertilizin", a substance which is secreted by the egg and which is necessary for fertilization. According to Lillie's theory [20, 21] fertilizin reacts with a receptor present on the surface of the spermatozoon. This reaction makes fertilizin capable of reacting with an egg-receptor and after the formation of the complex sperm-receptor–fertilizin–egg-receptor the egg is activated. The first part of this reaction corresponds to sperm agglutination.

Lillie also indicated the presence of another factor in the egg which he termed "antifertilizin". This factor was assumed to be liberated upon fertilization and to combine with the surplus fertilizin thus blocking the reaction between the sperm-receptors of supernumerary spermatozoa and fertilizin. This last reaction was postulated to serve as a block to polyspermy [20].

Though the fertilizin theory is based on many assumptions it has been and still is supported by many workers and especially Tyler has devoted much work to the development and extension of this theory [27–34].

However, the theory has also met much criticism [4, 6, 23, 24]. Loeb pointed out that fertilizin and the jelly layer are synonymous [23] which was later confirmed by Tyler *et al.* [28, 29, 31, 32], Hartmann *et al.* [14] and Vasseur [37, 38]. Loeb showed [23, 24] in contrast to Lillie [20] that eggs deprived of their jellies by treatment with acid sea water could still be fertilized. This result was later corroborated by Lillie [22] and several other workers [14, 29] though they found a decrease in the capacity of the eggs to become fertilized after removal of the jellies. That jelly-free eggs could be fertilized was, however, a fact definitely speaking against the fertilizin theory but this was overcome by making the assumption that there still remained a thin layer of fertilizin on the egg surface even after treatment

with acid sea water [30, 33]. This hypothetical layer, though not sufficiently potent to agglutinate the sperm was supposed to be sufficient to bring about fertilization [30, cf. 5].

Lillie's theory states that antifertilizin reacts with fertilizin and makes this latter substance unavailable for a reaction with the sperm-receptor. Antifertilizin is believed to block and impair fertilization [5, 14, 27, 34] to a certain extent. That preparations of antifertilizin may interfere with fertilization, seems to be obvious because antifertilizin in high concentrations causes cytolysis of the egg [9], which is also achieved if e.g. albumin is added in a high concentration. Moreover, a treatment of the jelly-free egg with antifertilizin in a moderate concentration would be expected to inactivate completely the hypothetical layer of jelly adhering to the egg surface, thus making the eggs unfertilizable.

However, the present author was able to demonstrate that a complete removal of the jelly layer very much improved fertilization [6]. The fertilization rate was also enhanced. Furthermore it was shown that treatment of normal eggs as well as of jelly-free eggs with antifertilizin in concentrations which bring about a strong precipitation of the intact jelly layer, markedly improves fertilization [9].

These results do not agree with the fertilizin theory nor with the results obtained by previous authors working with fertilization of jelly-free sea urchin eggs [14, 20, 29].

Vasseur [35] observed, when investigating the appropriate pH for jelly removal, that if the eggs were subjected to pH 4.0–5.0 a certain percentage of the eggs became cytolysed. This very important observation indicated that the mode of removal of the jelly layer was decisive for the fertilizability of the eggs. The present writer [6] investigated the influence of different concentrations of HCl on eggs from several sea urchin species by using the fertilization rate method [8, 9] and it was found that a short treatment at pH 5.8 had an entirely improving influence on fertilization. The supporters [30, 33] of the modified fertilizin theory might probably object that there still remained a molecular layer of jelly on the egg surface and that this hypothetical layer made the eggs still fertilizable. The fact that treatment with antifertilizin [9] improves and enhances fertilization instead of impairing or blocking it does not support the assumption of a molecular jelly layer adhering to the egg surface even after treatment with acid sea water. It is also difficult to understand why a thin submicroscopical jelly layer should improve fertilization more than does the intact jelly coat. The present author also carried out experiments [6] with removal of the jelly according to the

methods used by previous workers [28, 29, 32] and found that the fertilization rate was impaired if the eggs were subjected to pH 4.5–5.0 and at pH 3.5–4.0 the percentage of fertilizable eggs was only 20–40 even if the exposure to the acid medium was very short. The depressed fertilizability previously observed after jelly removal [14, 29, 30] must therefore be ascribed to an injury of the egg caused by too acid a pH and not to the loss of the jelly coat.

It has been reported [4, 10, 13, 19] that "egg water" (i.e. sea water containing dissolved jelly substance) acts by improving the capacity of the eggs to become cross fertilized. Especially are crosses with *Arbacia* sperm difficult to make and the addition of *Arbacia* egg water to the fertilization medium can effect an increase in the percentage of cross fertilized eggs [4, 10, 13, 19]. The results with jelly-free eggs referred to above suggested an investigation of the role of the jelly in cross fertilization experiments. In experiments with *Strongylocentrotus droebachiensis* and *Strongylocentrotus pallidus*, two closely related species from the west coast of Norway [cf. 36], it was observed that cross fertilization was more difficult to obtain [cf. 37 p. 17] than homologous fertilization. The present author found that the percentage of hybrids was increased if the jelly layers had been removed before insemination. In 1952 Harding and Harding [12] demonstrated that the percentage of cross fertilized eggs was very low when normal eggs from *Echinocardium cordatum* were inseminated with sperm from *Psammechinus miliaris*. However, after removal of the jelly coats the capacity for cross fertilization was very much increased.

The present investigation was carried out in Naples using *Psammechinus microtuberculatus* and *Paracentrotus lividus*. Some experiments were also made with *Echinocardium cordatum* and *Psammechinus miliaris* at Kristinebergs Zoologiska Station, Swedish west coast.

The jellies were removed by using the method previously described [6] and the fertilization experiments were performed according to the fertilization rate method [8 cf. also 6, 9].

EXPERIMENTS

The effect of jelly removal on hybridization.—Eggs from *Psammechinus microtuberculatus* were treated with acid sea water at pH 5.8 for 60–120 seconds [6]. The jelly-free eggs were tested in parallel fertilization rate experiments where insemination was performed either with *Psammechinus* sperm or with *Paracentrotus* sperm. The concentration of sperm was the same in both experiments and the number of sperm was 1–2 $\times 10^6$/ml, which is a rather low concentration. Similar experiments were carried out with the eggs with intact jelly coats.

In the experiments with untreated eggs the fertilization rate of the eggs inseminated

with heterologous sperm was considerably lower than that of the eggs inseminated with homologous sperm. The total number of cross fertilized eggs was as a rule lower than that of the control (cf. Fig. 1).

After removal of the jellies the rate of cross fertilization was very much increased and exceeded the rate of intact eggs inseminated with homologous sperm. These experiments demonstrate that the jelly coat *in situ* offers a general obstacle to fertilization [cf. 6]. It is also evident that the jelly is a potent inhibitor of cross fertilization.

Preliminary experiments were carried out with the other sea urchin species common in the Gulf of Naples. In none of these crosses did the removal of the jelly coat effect such a drastic improvement as that obtained in the crossing of *Psammechinus* female with *Paracentrotus* male. These experiments do, of course, not prove that the jelly is ineffective in preventing cross fertilization in these crosses but they just indicate that there also exist other barriers to cross fertilization [cf. 3, 18, 19].

At Kristineberg some fertilization rate experiments were made with the cross *Echinocardium cordatum* female *versus Psammechinus miliaris* male. During the summer 1955 the *Echinocardium* eggs showed a high resistance against homologous fertilization though the eggs seemed to be fully mature. After removal of the jellies the fertilization rate attained normal values.

In these experiments the rate of heterologous fertilization of jelly-free eggs exceeded that of the corresponding homologous insemination. The removal of the jellies also markedly increased the total percentage of fertilized and cross fertilized eggs (cf. Fig. 2). The number of sperm in these experiments was about 10^6 per ml.

It is obvious that the jelly coat of the *Echinocardium* egg constitutes an essential mechanism for protecting the egg against foreign sperm. After removal of the jelly the egg becomes very susceptible to heterologous insemination; as a matter of fact the somewhat higher fertilization rate of heterologous insemination of jelly-free eggs as compared with the homologous insemination of the same eggs seems to be significant and has returned in all the experiments. This difference might possibly be dependent upon a higher viability of the *Psammechinus* sperm.

The influence of "egg water" on fertilization.—It has been observed that addition of egg water (i.e. sea water containing dissolved jelly substance [cf. 37]) in some species increases the capacity for cross fertilization [4, 10, 13, 19]. Especially in crosses with *Arbacia* sperm [4, 10, 19] the addition of *Arbacia* egg water exerts a favorable influence. It has been claimed that it is only the paternal egg water which acts by improving cross fertilization [cf. 13], but in experiments with *Strongylocentrotus purpuratus* female × *Strongylocentrotus franciscanus* male Harvey [15] found that the addition of *S. purpuratus* egg water increased the percentage of hybrids.

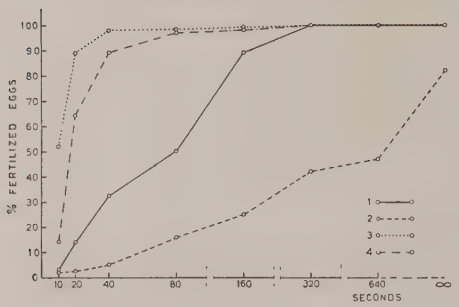
As was already mentioned jelly solution impairs fertilization when added in high concentrations [6 cf. 29]. Moreover, it has also been demonstrated that the concentration of egg water added in cross fertilization experiments might not be too high when the improvement is changed into an inhibition [10].

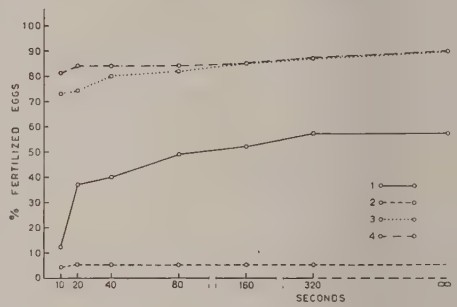
Many workers have tried to correlate the degree of cross agglutination with the ability for cross fertilization [4, 21, 22, 23, 24] but no closer relationships has been observed.

In order to examine the influence of maternal and paternal egg water cross fertilization experiments were made according to the fertilization rate method [8]. Instead of egg water, which is subject to bacterial infection during the course of prepara-

tion, very dilute jelly solutions were used because it is easier to determine the concentration of jelly solution than of egg water and because it is desirable to work at the same concentration of high molecular substance. The jelly solutions used contained about 0.03 per cent of high molecular substance (for methods of preparation cf. [6]). Before use the pH of the jelly solutions was adjusted to 8.0.

The results revealed that diluted jelly solution acts differently on intact eggs and on jelly-free eggs and also on homologous fertilization and on cross fertilization. It has already been shown that the addition of jelly solution in high con-





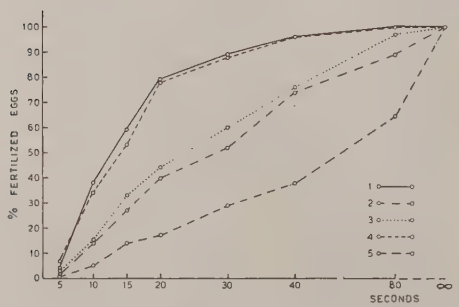


Fig. 1 (*top, left*). Eggs from *Psammechinus microtuberculatus*. The concentration of sperm was 1×10^6 per ml.
1, Eggs with intact jelly coats inseminated with *Psammechinus* sperm. *2*, Eggs with intact jelly coats inseminated with *Paracentrotus* sperm. *3*, Jelly-free eggs inseminated with *Psammechinus* sperm. *4*, Jelly-free eggs inseminated with *Paracentrotus* sperm.

In all the figures of the present paper each circle of the curves represents 300 counted eggs.

In all the experiments of this investigation the interaction between sperm and eggs was interrupted at different intervals of time (5, 10, 20 etc. seconds) after insemination by adding sodium lauryl sulphate to a final concentration of 0.001 per cent.

Fig. 2 (*top, right*). Eggs from *Echinocardium cordatum*. The concentration of sperm was 1×10^6 per ml.
1, Eggs with intact jelly coats inseminated with *Echinocardium* sperm. *2*, Eggs with intact jelly coats inseminated with *Psammechinus* sperm. *3*, Jelly-free eggs inseminated with *Echinocardium* sperm. *4*, Jelly-free eggs inseminated with *Psammechinus* sperm.

Fig. 3 (*left*). Jelly-free eggs from *Paracentrotus lividus*. The concentration of sperm was 2×10^6 per ml. Sperm from *Paracentrotus*.
1, Control. *2*, 5 ml of jelly solution from *Paracentrotus* were immediately before insemination added to 125 ml of egg suspension. *3*, 5 ml of jelly solution from *Psammechinus microtuberculatus* were immediately before insemination added to 125 ml of egg suspension. *4*, To 125 ml of egg suspension 5 ml of jelly solution from *Arbacia lixula* were added immediately before insemination. *5*. To 125 ml of egg suspension 5 ml of jelly solution from *Sphaerechinus granularis* were added immediately before insemination.

centrations to jelly-free eggs effects a more pronounced retardation of the fertilization rate of these eggs than does the addition of the same amount of jelly solution to intact eggs with jelly coats (cf. [6] Fig. 1). A reasonable explanation of this phenomenon seems to be that the addition of jelly solution to intact eggs implies a relatively small change in the total concentration of jelly since it must be difficult to surpass the concentration of jelly represented by the jelly coat *in situ*. The jelly-free eggs have no such barrier and therefore the inhibiting action of the jelly substance can display itself and is manifested by a considerable decrease in the fertilization rate.

It seemed desirable first to make a comparison between the action on homologous fertilization of jelly solutions from the sea urchin species common in the Gulf of Naples in order to determine if any species differences exist. The jelly solutions used contained about the same amount of high molecular substance and a typical experiment is shown in Fig. 3. The differences with respect to inhibiting capacity are rather striking, and the failure of *Arbacia* jelly to inhibit the fertilization rate is of especial interest. Furthermore, the inhibition exerted by the jelly solutions seems not to be species-specific.

The action of jelly solution on cross fertilization.—Experiments were made with *Psammechinus microtuberculatus* eggs and sperm from *Psammechinus* or *Paracentrotus* and addition of very dilute jelly solutions. With intact eggs the influence of jelly solution on the fertilization rate after homologous insemination was very weak. There was a slight retardation both with *Psammechinus* and *Paracentrotus* jelly. After cross insemination the paternal jelly showed a weak increase in the rate when the maternal jelly gave a slight inhibition. The differences from the control were within ± 10 per cent. (For the significance of these differences cf. [17].)

The same type of experiments was also made with jelly-free eggs. After homologous insemination dilute *Psammechinus* and *Paracentrotus* jelly solution had almost no effect on the fertilization rate. Only in a few experiments did the paternal jelly cause a slight improvement. With jelly solution from *Arbacia*, however, there was an obvious increase in the rate (cf. Fig. 4a).

After cross insemination, on the other hand, a general improvement was observed after addition of jelly solutions from the three species mentioned above (cf. Fig. 4b). The improvement with *Psammechinus* jelly was perhaps a little weaker than that obtained with *Paracentrotus* or *Arbacia* jelly though the differences were not very accentuated.

The results recorded after addition of jelly solutions in cross fertilization experiments do not seem to indicate any "fertilizin-action" of the jelly (cf. the improvement with *Arbacia* jelly in Figs. 4a and b). On the contrary the action of the added jelly seems to be quite nonspecific, but the jelly initiates an obvious activation of sperm motility which might be of importance in cross fertilization experiments. The present writer has also observed that heparin, which is chemically related to jelly substance, effects an activation of sperm motility. In high concentrations heparin blocks fertilization in analogy with jelly solution [11].

The positive effect obtained with jelly solution from *Arbacia* on homologous and heterologous fertilization of *Psammechinus* eggs suggested experiments with heparin in order to investigate if this substance in low concentration has a similar promoting action.

The results revealed that heparin is almost as effective in promoting cross fertilization as jelly solution. It is, however, rather difficult to find the optimal concentration for improvement with heparin because the susceptibility of the gametes from different specimens varies. This is also valid for the action of jelly solution and there-

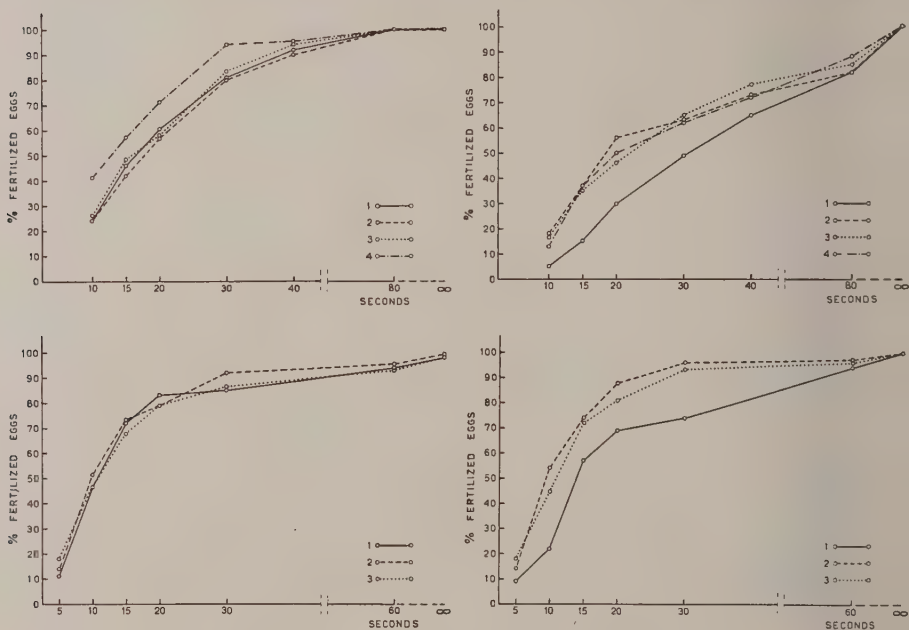


Fig. 4 *a* (*top, left*). Jelly-free eggs from *Psammechinus microtuberculatus*. Sperm from *Psammechinus*. Concentration of sperm 10^6/ml. The jelly solutions used were immediately before insemination added to 100 ml of egg suspension.
1, Control. *2*, 1 ml of jelly solution from *Paracentrotus*. *3*, 1 ml of jelly solution from *Psammechinus microtuberculatus*. *4*, 1 ml of jelly solution from *Arbacia*.
Fig. 4 *b* (*top, right*). Jelly-free eggs from *Psammechinus microtuberculatus*. Sperm from *Paracentrotus*. Concentration of sperm 10^6/ml. The jelly solutions used were added to 100 ml of egg suspension immediately before insemination.
1, Control. *2*, 1 ml of jelly solution from *Paracentrotus*. *3*, 1 ml of jelly solution from *Psammechinus*. *4*, 1 ml of jelly solution from *Arbacia*.
Fig. 5 *a* (*bottom, left*). Jelly-free eggs from *Psammechinus microtuberculatus*. Sperm from *Psammechinus*. Concentration of sperm 2×10^6/ml.
1, Control. *2*, 1 ml of jelly solution from *Psammechinus* added to 100 ml of egg suspension immediately before insemination. *3*, Heparin added to a final concentration of 0.0005 per cent immediately before insemination. (Heparin from *Vitrum*, lot 5–31.)
Fig. 5 *b* (*bottom, right*). Jelly-free eggs from *Psammechinus microtuberculatus*. Sperm from *Paracentrotus*. Concentration of sperm 2×10^6/ml.
1, Control. *2*, 1 ml of jelly solution from *Psammechinus* added to 100 ml of egg suspension immediately before insemination. *3*, Heparin added to a final concentration of 0.0005 per cent immediately before insemination.

fore it is desirable to make an orientating experiment in order to settle the suitable range of concentration which will give improvement or inhibition. Figs. 5a and 5b show that the concentration of heparin (or jelly solution) which gives an evident improvement of heterologous fertilization does not at all affect homologous fertilization.

Results similar to those obtained with heparin have also been observed with dextran–sulphuric acid, chondriotin–sulphuric acid and alginic acid.

DISCUSSION

The present results show that the jelly coat in some species of sea urchins represents the main barrier to heterologous fertilization. In other species the effect of removing the jelly coat is not so striking, and this indicates that there are still other mechanisms involved in protecting the sea urchin egg against foreign sperm. As was already shown the species-specific structures may be removed by treating the egg with trypsin or isotonic urea solution [3, 18, 33]. The facilitating effect of such treatments is, however, restricted to certain combinations of crosses [18, 19]. The vitelline membrane has been credited with the role of protecting the egg against heterologous insemination and this seems to be the case in a number of species [3, 18, 33]. However, in other species the vitelline membrane is not the site of the species-specificity ([12], present investigation) because it is possible to cross fertilize even in the presence of this membrane.

Bohus Jensen [2] reported that treatment of the eggs with trypsin interferes with homologous fertilization (cf. also [33]). Even a pretreatment of the eggs with concentrations of the enzyme which facilitate cross fertilization brings about a depressed susceptibility of the eggs to the homologous sperm [3]. This most interesting observation indicates that the concentrations of trypsin used to increase the capacity for cross fertilization produce a severe injury to the egg and the normal mechanism of fertilization. Consequently, it is doubtful if this mechanism is involved in the engulfing of heterologous sperm after treatment with trypsin. As was pointed out by Bohus Jensen [3] the procedures used to obtain cross fertilization do not exclude the possibility that this treatment also changes more deeply lying structures, thus making the egg cross fertilizable.

It is well known that it is necessary to use higher concentrations of sperm to obtain cross fertilization than to get homologous fertilization [cf. 4]. The number of sperm in e.g. the experiments reported by Harding and Harding [10] is likely to have been about 10^8/ml which concentration if used in homologous insemination would induce heavy polyspermy or even cytolysis

[cf. 7]. Furthermore, it was recently demonstrated that the sperm medium contains substances influencing fertilization [16]. The action of these factors must also be kept in mind in cross fertilization experiments if the concentration of sperm is high. Therefore, the present writer prefers to use normal concentrations of sperm, i.e. about 10^6/ml when the influence of the factors introduced with the sperm is negligible.

In previous work the mechanism blocking heterologous fertilization has mainly been ascribed to the egg and the possibility of such a mechanism on the part of the sperm has been rather much overlooked. However, it seems feasible to the present writer that there is also a species-specific mechanism of the sperm and that this mechanism might be concerned with the capacity of the sperm for initiating the cortical reaction (cf. [1]). The cortical breakdown and membrane formation are as a rule not proceeding in a perfect manner after cross insemination and many cortical granules remain intact. Substances favoring membrane elevation and facilitating the cortical propagation also improve heterologous fertilization [1, 10]. The results with e.g. $NaIO_4$ in combination with egg water [10] are of particular interest and suggest that the threshold for activation of the egg is lowered by $NaIO_4$ [1] and that this effect together with the influence of $NaIO_4$ [8] and egg water on the sperm facilitates the engulfing of the foreign spermatozoon.

From the present results it is seen that the improvement in the fertilization rate upon addition of jelly solution in cross fertilization experiments is considerable. However, the final number of fertilized eggs is not influenced because jelly-free *Psammechinus* eggs as a rule give 100 per cent fertilization when inseminated with *Paracentrotus* sperm. Pretreatment of the eggs with jelly solution does not at all influence the fertilization rate and the action of jelly solution seems to be concerned with an activation of the sperm. The fact that heparin shows an action analogous to jelly solution strongly indicates that the influence of this latter substance must be related to its high contents of acid polysaccharide. Vasseur suggested [35, 37] that the sperm activating effect observed after addition of jelly solution depends upon strongly charged groups in the polysaccharide part of the jelly, which groups are already exposed in the untreated jelly solution but which become still more exposed after moderate autoclaving [39]. The high activating effect obtained with *Arbacia* jelly solution supports this view as *Arbacia* jelly has a high content of ash and sulphur [35]. Further evidence for this interpretation was produced by Elster [4] who investigated the influence of egg water on cross fertilization in a number of species but in none of these crosses did he obtain a promoting effect as manifest as with *Arbacia* egg water and *Arbacia* sperm.

Though no objective methods are available for measuring sperm motility the data presented above suggest that the enhanced motility seen upon addition of jelly solution or heparin is correlated with the favorable influence on heterologous fertilization. The sperm stimulating activity of jelly solution is entirely nonspecific [13, 40], as it might be replaced by e.g. heparin and thus no "fertilizin-reaction" can be involved. The inhibiting action of *Arbacia* jelly on the fertilization rate is low (cf. Fig. 3) which probably indicates that the sperm stimulating effect predominates.

Because of the injurious action of jelly solution on membrane elevation [25, 26] and the promoting influence of jelly removal on the state of the membrane [6, 7] the action of jelly solution in cross fertilization experiments seems to be concerned with a species-nonspecific activation of the sperm. The increase in motility probably reflects an activation of some enzyme system (or systems) in the sperm necessary for initiating the cortical breakdown [cf. 1].

The results presented do not indicate that the origin of the jelly is decisive for the improvement of cross fertilization. However, it seems quite possible to the writer that in some species the sperm "prefer" egg water from the homologous species because the chemical composition of the jelly coat is fairly species-specific [35, 37].

SUMMARY

The jelly coat represents in *Psammechinus microtuberculatus* and *Echinocardium cordatum* a strong barrier to heterologous fertilization.

The inhibiting action on fertilization exerted by jelly solutions is not species-specific.

Very dilute jelly solutions (= egg water) enhance the fertilization rate of jelly-free eggs inseminated with heterologous sperm. This effect of jelly solution does not seem to be species-specific.

The addition of dilute jelly solution to eggs with intact jelly coats and inseminated with homologous or heterologous sperm does not markedly influence the fertilization rate. This is probably explained by the fact that the eggs are already surrounded by "egg water" (= the jelly coat).

Heparin was found to exert an action similar to that of jelly solution.

Heparin and jelly solution are only effective when present at the time of insemination and they both increase the motility of the spermatozoa.

My sincere thanks are due to Professor John Runnström for his unfailing interest in this work. I also wish to express my gratitude to Drs. R. and P. Dohrn, Naples,

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ISOLATION OF PURE AND UNALTERED LIVER NUCLEI MORPHOLOGY AND BIOCHEMICAL COMPOSITION

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Every technique of isolation of a cell component should attempt to satisfy the following conditions: preservation of its morphology and its biochemical composition, homogeneity of the fraction and a sufficiently high yield. When isolating nuclei by differential centrifugation, the above principles led to the selection of some methods which have been recently discussed [1]. Those proposed by Hogeboom, Schneider and Striebich [2] and by Dounce [1] showed clearly that the contamination of the nuclei by cytoplasmic structures is one of the main difficulties encountered in the isolation of the nuclear fraction. Indeed intact cells, mitochondria, connective tissue and erythrocytes may be found in this fraction, and their presence, even in very small quantities, has an important effect on the nuclear composition. The calculations show that the method of Hogeboom *et al.*, which leaves in the nuclear fraction < 1 per cent of intact cells and < 0.5 per cent of mitochondria of the initial homogenate, gives nuclei in which about 8.5 per cent of nitrogen is of extranuclear origin [2].

If we consider the principle of differential centrifugation which is based on the sedimentation rates, it is obvious that this contamination is theoretically impossible to avoid, since all the particles migrate in the same direction. Purification can only be made by washing several times and by using processes such as double perfusion of the liver, filtration of the homogenate, use of $CaCl_2$, adjustment of pH, etc. [1, 2]. Despite these precautions, intact cells and mitochondria can never be completely eliminated and an accurate count of contaminants is necessary, especially in the study of the nuclear composition [2].

On the other hand, the use of the differences of density between the particles enables a complete separation to be made, since only particles having a density higher than that of the medium sediment out during the centrifugation, while the others migrate in the opposite direction. A single centrifugation separates the densest particles if the density of the medium is suitably fixed. This principle has been very little used; since the publication

by one of us [3], we can only cite, to our knowledge, the preliminary note of Krakaur *et al.* [4] which mentioned such a method, but there has been no subsequent publication of the exact conditions. Applying this principle to the separation of rat liver nuclei, the density of which is about 1.4 whereas that of the intact cells and cytoplasmic components varies from 1.1 to 1.25, we obtain in a yield of 45 to 55 per cent, *nuclei completely free from cytoplasmic contaminants and morphologically very well preserved.* The results given in this paper relate to normal rat liver, but the technique has been applied to tumour-bearing liver and also to kidney.

Method of isolation

The rats are kept fasting for 18 h. before killed by bleeding. All subsequent operations are carried out at 4°C. The liver is weighed, cut in small pieces and transferred with 20 vol. of 2.2*M* sucrose solution (d. = 1.28) to a plastic Potter's homogenizer immersed in melting ice. About 2 or 3 up- and downstrokes are generally required for a suitable homogenization (60–80 seconds for 40 cc solution). The homogenate is centrifuged for 60 minutes at 40,000 *g* in an International refrigerated centrifuge (acceleration calculated for the bottom of the tube). Whereas the intact cells, connective tissue, mitochondria and erythrocytes migrate to the top, all the nuclei in the homogenate sediment and form a thin pellet looking like a colorless jelly. They are not agglutinated when resuspended in the sucrose solution.

Morphology

Microscopic examinations of a suspension of nuclei and a thin section of the sediment prove the purity of the preparations (Figs. 1, 2). Systematic examinations enable us to confirm that there are never any intact cells or erythrocytes, according to theory. The only impurities are a few strands of connective tissue. Also less than 10 per cent of nuclei may show a ruptured membrane and fragments of nucleoplasm enclosing nucleoli are recognizable.

Electron microscopic examination of ultra-thin sections of the sediment.— The electron micrographs show that the morphological integrity of isolated

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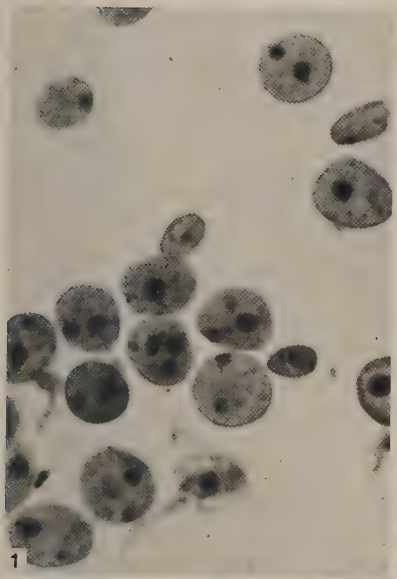
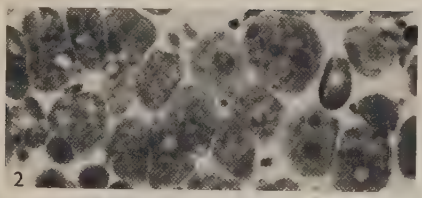
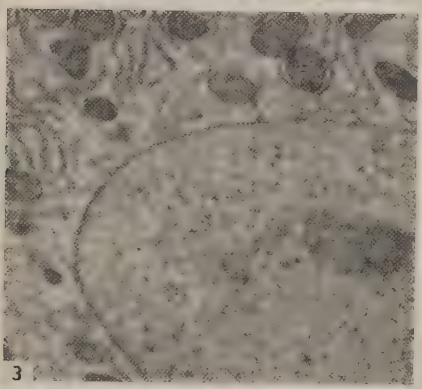
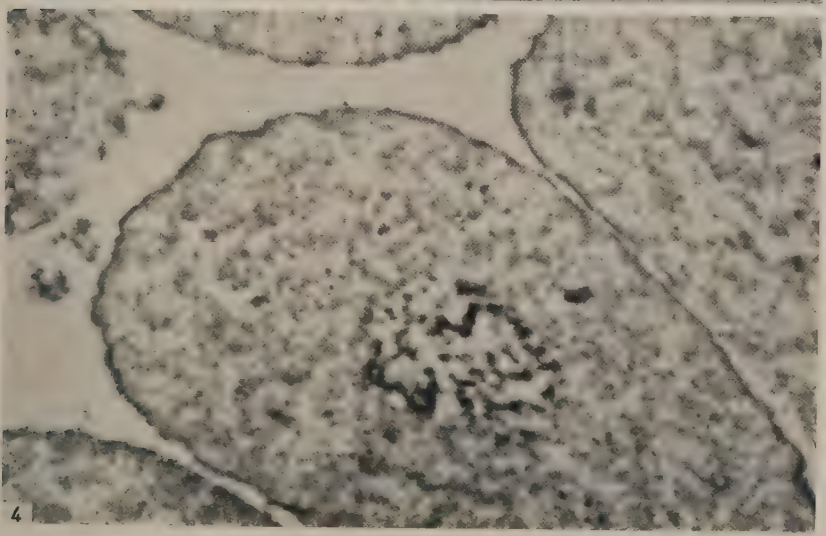
Fig. 1. Suspension of isolated nuclei in 2.2*M* sucrose solution, stained with methylene blue. Magnification: ×1200.
Fig. 2. Semi-thin section of the sediment of nuclei imbedded in butyl-methacrylate and stained with Regaud's hematoxyline. Magnification: ×1200.
Fig. 3. Electron micrograph of a portion of liver cell. Magnification: ×10,000.
Fig. 4. Ultra-thin section of the sediment of nuclei, fixed in 1 per cent osmium tetraoxyde buffered at pH 7.4 and imbedded in butyl-methacrylate. Magnification: ×12,000.









nuclei is preserved even at the level of the infrastructure (Fig. 4). If they are compared with Fig. 3 which represents a portion of a liver cell, they show a very good state of preservation, despite the time-interval between the death of the rat and the fixation with osmium tetraoxyde (80 minutes). This may be seen by the persistence of the nuclear membrane, the nucleolar structure and the homogeneity of the nucleoplasm. Systematic examinations confirm the purity of the preparations and the total absence of mitochondria and ergastoplasmic structures. Occasionally nuclei are ruptured.

Biochemical composition of nuclei

This has been determined by Schmidt-Thannhauser's technique, in which trichloroacetic acid has been replaced by perchloric acid [5]. The homogeneity of the results obtained with these preparations proves that the method is reproducible. Moreover, the sufficiently high yield leads us to think that the isolated nuclei are representative of all the nuclei of the tissue. The purity of the preparations allows us to calculate without correction, the ratios between the different constituents.

1. Calculated in μg phosphorus per mg of protein nitrogen, the results show the low concentration of phospholipids in the nucleus (4 5 times smaller than in the cell), whereas that of RNA is only a little smaller than that of the total liver tissue. Moreover the ratio μg DNA P/mg PN is 123 (s. d. = 7) in our preparations; by relating μg DNA P to mg of total nitrogen

Table I

	Lipid phosphorus		RNA P		DNA P	Proteins
	μg per mg PN	μg per μg DNA P	μg per mg PN	μg per μg DNA P	μg per mg PN	μg per μg DNA P
Homogenate	60	5.5	27	2.7	11	
Nuclei	13.3	0.11	19.7	0.16	123	40.1
Standard deviation . .	1.4	0.011	1.7	0.017	7	2.7
	(16)	(17)	(20)	(19)	(18)	(18)

The figures in brackets represent the number of observations.
RNA P: Ribonucleic acid phosphorus (Mejbaum's method).
DNA P: Desoxyribonucleic acid determined by spectrophotometry.
PN: Protein nitrogen is determined by estimating the N content of the alkaline digest after acid-soluble and lipid fractions are removed.
Proteins: (Protein nitrogen minus nucleic nitrogen) × 6.25.

and after taking into account cytoplasmic contaminants, Hogeboom *et al.* obtained a corrected value of 100 [2].

2. Calculated in μg P or N per μg of DNA P, the ratios enable us to refer to the true cellular constant. Our results show that the RNA P corresponds to 16 per cent of the DNA P. The most recent values found in literature show that RNA represents about a quarter of the DNA. The differences between these figures and ours may be explained, in our opinion, by the complete lack of cytoplasmic contamination in our preparations.

The calculations made for a yield of 100 per cent of nuclei show that the nuclear protein nitrogen is about 10 per cent of total protein nitrogen, the lipid phosphorus is 2–2.5 per cent of total lipid phosphorus and the RNA P is 6–7 per cent of total RNA P, in the diet of our rats.

It should be recalled that with regard to the protein-bound phosphoric esters, it has been shown by one of us [6] that there is a large amount of these compounds in the nuclear fraction. Whereas in the total liver tissue, they represent one third of the RNA phosphorus, in the nuclei, ester phosphorus is equal to, if not greater than ribonucleic acid phosphorus.

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THE TOTAL CONTENT OF ASCORBIC ACID IN THE DEVELOPING SEA URCHIN EGG

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Changes in easily oxidized and reduced substances may be correlated with determination and differentiation processes in the sea urchin egg. As an introduction to an investigation of such changes, analyses of the total amounts of some of these substances have been carried out. The present work is concerned with the content of ascorbic acid in different stages of development.

MATERIAL AND METHODS

The analyses in this investigation were carried out mainly on lyophilized sea urchin eggs and larvae of *Paracentrotus lividus*, collected at the Station Biologique de Roscoff, Finistère, France. Eggs from many females were mixed in sea water, fertilized in a large beaker and cultivated in glass trays, as described by Lindahl [7]. The temperature was kept constant at 22°C, and at certain stages samples were taken for analysis. The eggs were washed in sea water, collected by centrifugation, rapidly frozen and lyophilized. The dried material was ground in a mortar and stored over phosphorus pentoxide in a desiccator at +4°C until used.

Extracts for the ascorbic acid analyses were prepared as follows. 100 mg dry powder was suspended in 2 ml ice-cold distilled water in an ice-cold test tube, dipped into a freezing mixture of NaCl and ice. By repeated freezing and thawing of the suspensions during continuous stirring with a glass rod, the powder was triturated by the paste of ice crystals, and a homogenate was obtained. This homogenate was then treated with cold 5 per cent trichloracetic acid (TCA) for 48 hours in a refrigerator and an extract was collected by centrifugation [11]. The results obtained with this method were not quite reproducible, however, and it was found more favourable to treat the homogenates with hot TCA, or sometimes with metaphosphoric or sulphosalicylic acids. The ascorbic acid was also fractionated according to Sumerwell and Sealock [12]. By deproteinizing the homogenates with 95 per cent ethyl alcohol an extract of free ascorbic acid was obtained, after which a bound form was released upon treatment of the residue with hot 5 per cent TCA.

The clear extracts were finally analyzed for ascorbic acid according to the Roe and Kuether colorimetric 2,4-dinitrophenyl-hydrazin method [11]. The intensity of the colour was determined in a Spekker photometer. The method indicates the sum of ascorbic, dehydroascorbic, and diketogulonic acids, here simply called ascorbic

acid. The amounts of ascorbic acid in the extracts were referred to the Kjeldahl nitrogen value (N) of the lyophilized powders. The nitrogen determinations were carried out as described by Gustafson and Hjelte [2].

RESULTS

The total content of ascorbic acid during early sea urchin development is represented by curves in Figs. 1 and 2. After fertilization the amount decreases somewhat. From the present material it is impossible to determine

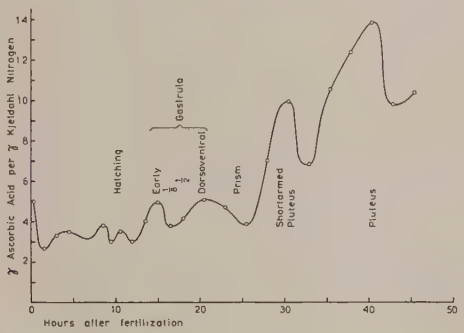

Fig. 1. Total content of ascorbic acid during sea urchin development.

exactly when this reduction occurs. The following period is characterized by a series of small increases, which occur during late cleavage stages, just before ciliar activity starts, at hatching, and during gastrulation. These increases are alternated with small decreases, so that the values from the time of fertilization until the end of gastrulation (about 25 hours after fertilization) vary between 3 and 5 γ per γN. Following gastrulation the content of ascorbic acid strongly increases to a high level, after which wavelike variations occur, which can be correlated with different morphogenetic processes during late differentiation of the larva. The amount of ascorbic acid then reaches values of 10 and more γ per γN.

The results from different batches of eggs are almost identical in regard to the early part of development. In the latter part, however, the increase in ascorbic acid and the number of waves often differs (cf. Figs. 1 and 2). In the batch represented in Fig. 2 with a lower rise, the arms of the plutei were poorly developed, whereas plutei with well developed arms were present in the other batch (Fig. 1). It is thus obvious that the differences between the curves are correlated with morphological differences between the batches of eggs.

Fractionated extraction of ascorbic acid revealed the existence of the acid both in the free and in the bound state. The free, i.e. the alcohol-extracted, fraction is present in an amount varying within very narrow limits (2–4 γ per γN) during the whole development. The bound fraction, released by treatment with warm TCA, is, however, subjected to drastic changes. From fertilization to the early gastrula stage the amount decreases slowly from 1 γ

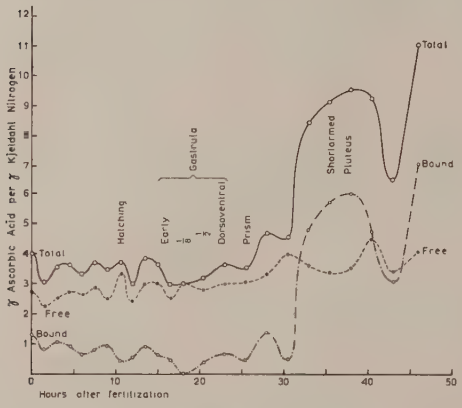

Fig. 2. Free, bound and total ascorbic acid during sea urchin development.

per γN to zero. After gastrulation rapid synthetic and degradative processes alternate and maximum values of 7 to 8 γ per γN are reached. The cyclic changes in the total ascorbic acid content, which are correlated with the late differentiation processes of the larvae, are thus mainly due to changes in the amount of bound ascorbic acid (cf. Fig. 2).

Some experiments were made concerning the extractability of the bound ascorbic acid. Ascorbic acid cannot be split off from the compound by treatment with cold water or 1*M* KCl. Part of it is released by a 45-minute treatment with hot water and by a 48-hour treatment with cold TCA. By hot TCA, metaphosphoric and sulphosalicylic acids, maximum amounts of bound ascorbic acid are brought into solution after some 10 minutes of treatment.

DISCUSSION

In the above study the total ascorbic acid content has been determined during different developmental stages of the sea urchin egg. Neither the present method nor the present material (lyophilized powder) permit a separation of reduced, i.e. ascorbic, acid and oxidized, mainly dehydro-

ascorbic, acid. It is therefore too early to judge the role of ascorbic acid for maintaining a reduced state in the sea urchin egg. A certain parallelism exists, however, between the amount of reduced glutathione and the sum of free ascorbic acid (ascorbic plus dehydroascorbic acid) in the sea urchin [1]. A similar agreement has often been found between reduced ascorbic acid and SH compounds [6, 14]. It is most likely, therefore, that a great part of the free ascorbic acid is present in the reduced state in the sea urchin.

Bound ascorbic acid, often called ascorbigen, has been observed both in plant and animal material [4, 9, 12, 13]. By hydrolysis of the complex compound reduced ascorbic acid is found to be released [13]. The component to which ascorbic acid is bound, is different in different material. Ascorbigens of animal origin have been found to contain a protein moiety [4, 12]. In view of the experiments on extractability reported above, ascorbigen is probably composed in the same way in the sea urchin embryo.

During the stages up to the end of gastrulation, the cyclic changes in the curves representing the total ascorbic acid, bound and free, are in good agreement with the periods of synthesis and degradation of the proteins and amino acids found by Kavanau [5]. Thus periods of high amounts of ascorbic acid coincide with periods of high amounts of free amino acids. During the postgastrula development, the ascorbic acid reaches very high values. These large increases of ascorbic acid occur simultaneously with the period of rapid development of coelom and skeleton in the pluteus larva, and also with the final differentiation of the intestine, which is to a great extent based upon processes in the layers derived from the mesenchyme. A similar wave of increase and decrease in the ascorbic acid contents in the chick embryo was found to coincide with the period of its most rapid growth [10]. These observations are in keeping with the common occurrence of a high ascorbic acid content in tissues during growth of mesoderm material and formation of intercellular substances [8]. The animalizing effect of the ascorbic acid analogue, glucoascorbic acid, supports the idea that ascorbic acid plays a role in the development of structures in the vegetal trend of development [3]. It is thus obvious that ascorbic acid takes a prominent part in the general metabolism of the sea urchin embryo.

SUMMARY

1. The total amount of ascorbic acid in the sea urchin egg varies on a relatively low level from the time of fertilization to the end of gastrulation, after which a sharp increase occurs, followed by wave-like changes.

2. A free, i.e. alcohol- or water-soluble, and a bound, probably protein-bound, component of ascorbic acid were separated. The free component varies at about the same level during the whole development. The bound component is very low until the end of gastrulation, after which an increase occurs.

3. The role of ascorbic acid in the general metabolism and for the maintenance of a reduced state in the sea urchin egg is discussed in relation to the results presented above.

The author wishes to express his most cordial thanks to Professor John Runnström and to Dr. Tryggve Gustafson for the great interest they have shown in this work. He also owes a great debt of gratitude to the authorities at Roscoff, France.

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REFRACTOMETRY OF TISSUE SECTIONS BY PHASE-CONTRAST MICROSCOPY

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In recent years, several authors have described the measurement of the refractive index of histological material by phase-contrast microscopy [10, 11, 9, 7, 8]. The principle has been to examine sections, by phase illumination, in a series of mountants of known refractive index. If the refractive index (RI) of the cell exceeds that of the mountant, the passage of light is relatively retarded by the cell, which then appears dark, or "phase-positive", against a paler background. Conversely, if the RI of the cell is less than the RI of the mountant, light is relatively accelerated in passing through the cell, which then appears pale, or "phase-negative", against a darker background. If the refractive indices coincide, there are no changes in phase; consequently there is no phase contrast and the cell is more or less invisible. The same principle has also been applied to the living cell by Barer [2, 3], Barer and Ross [4] and Barer *et al.* [5, 6, 7, 8].

There have been surprisingly few descriptions of critical investigations upon the refractive index of fixed tissue sections. The present experiments were undertaken to explore the potentialities of the technique, and the results are outlined in the following paper. A preliminary report of some of our findings was presented at the VIth International Anatomical Congress [16].

MATERIALS AND METHODS

The small intestine of the mouse was used for most of the experiments, the tissue being fixed in a variety of the common solutions. Additional work was carried out upon other organs and with other species, and upon tissue prepared by freeze-drying and freeze-substitution. The tissues were embedded in soft wax, m.p. 45°C, to avoid shrinkage, and the blocks were cut in a cryostat. It is obviously important, when comparing the phase appearance of different sections, to maintain their thickness as constant as possible. To this end, samples of different organs, and of the same organ when fixed in different ways, were blocked and cut together. Thin sections (2–3 μ) were mounted as usual on slides and their phase-contrast appearances studied in a manner similar to that described by Bennett *et al.* [9], as follows.

A section was first deparaffinized in xylol and then mounted in a fluid of known

RI. A coverslip was applied and a particular field chosen and marked for study with the 2 mm objective. After careful visual examination, the chosen field was photographed. The slip was taken off and the mountant washed off in xylol. The section was then mounted in another mounting fluid of known RI; the coverslip was re-applied and the chosen field again examined and photographed. This process was repeated until the desired range of RI had been covered. It entailed many observations on the same field. For instance, the most useful range was found to be from RI 1.5004 to RI 1.6192. This was covered by ten mountants, each constituting separate steps. On occasion, additional media were used.

The mountants were fluids supplied by British Drug Houses Ltd. for the measurement of RI. The following were employed in the present experiments:—

Ethyl bromide, 1.4239; liquid paraffin (A), 1.4721; benzene, 1.5004; ethyl iodide, 1.5133; chloro-benzene, 1.5243; methyl salicylate, 1.5362; ethylene bromide, 1.5385; nitro-benzene, 1.5521; bromo-benzene, 1.5600; benzyl benzoate, 1.5692; aniline, 1.5857; iodo-benzene, 1.6192; quinoline, 1.6232; and bromo-naphthalene, 1.6567.

A standard phase-contrast microscope (Cooke, Troughton and Simms Ltd.) was used. Photographs (9 × 12 cm) were taken in serial order and, for each mountant, the light transmission of various tissue constituents was compared with that of the background. Initial attempts to do this photometrically were not successful, and visual judgments were therefore made.

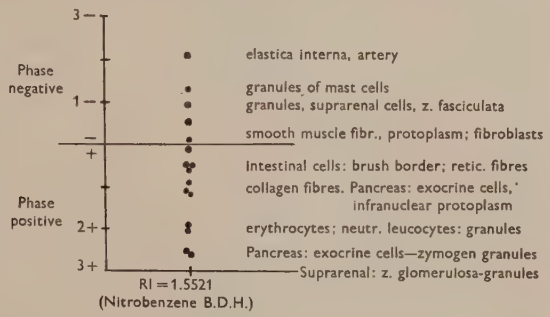
Some simple experiments were made with sections mounted on slides. After examining them as described above, they were transferred to water, or to water acidified with acetic acid to mimic staining in haematoxylin, then re-examined in the various mounting media after dehydration in alcohol.

Certain pitfalls and difficulties became apparent in the early stages of the work. It was found, for instance, that misleading results could arise through the incomplete permeation of the section by mounting fluid. Certain structures, such as nuclear membranes, zymogen granules and erythrocytes, were penetrated much more slowly than, for instance, cytoplasm and brush borders. This difficulty was overcome by waiting until the phase appearances were seen to have stabilized in one mountant before passing on to the next. Also, there was some difficulty with what may best be described as "glistening particles". When unstained, deparaffinized sections were examined by phase illumination, small, glistening particles were sometimes observed, especially around and upon nuclei, erythrocytes, etc. These glistening particles could be discharged by washing sections in water, and their nature is uncertain. Nedzel [21] described what seem to be similar structures; he regarded them as consisting of undissolved wax.

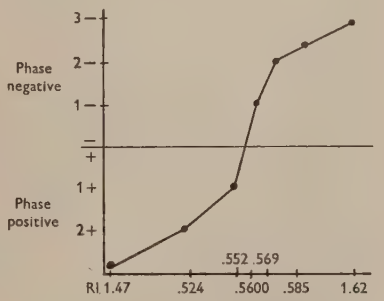
RESULTS

Graphic representation of phase appearances.—The phase appearances of a given tissue constituent in a given mountant were scrutinized from two points of view: first, to ascertain whether the object was darker or paler than the background, i.e. whether phase-positive or phase-negative; second, to determine the degree of contrast, i.e. to what extent it was darker or paler than the background.

A vertical scale was constructed (Text-fig. 1) with a midpoint corresponding to zero contrast. Above were three subjective values of increasing negative contrast, and below three similar values of increasing positive contrast. The visual appearances were then expressed as points along the scale. For example, the phase appearance of three fields when mounted in a medium,



Text-fig. 1. Optical differentiation of tissues from a mouse after fixation in Helly, when mounted in nitro-benzene. (Compare figs. 1–3.)

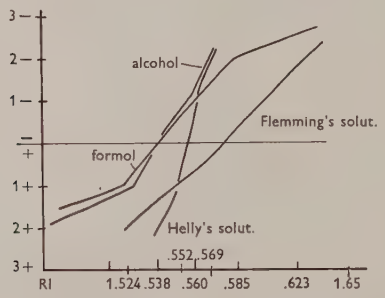


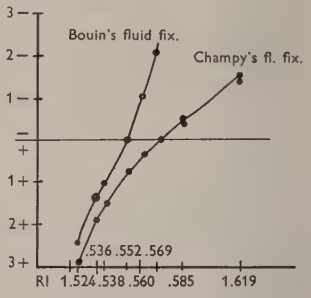
Text-fig. 2. Optical differentiation and refractometric value of brush border of cat intestinal epithelial cells. (Compare figs. 4–10.)

RI 1.5521, are shown in Figs. 1, 2 and 3. It will be noted that the elastica interna of the artery visible in Fig. 1 was very much paler than the background and showed a marked degree of phase-negative contrast. Granules of tissue mast cells (Fig. 2) transmitted more light than the background, but the contrast was not so striking as in the former case. The positions given to these structures on the scale is shown in Text-fig. 1; a higher negative value was allotted to elastica interna than to mast cell granules. Zymogen granules of the pancreas (Fig. 3) were very much darker than the background and were allotted the maximum value for positive contrast (Text-fig. 1). Erythrocytes were not so dark, and the cytoplasm of acinar cells less dark still (Text-fig. 1).

Measurement of RI.—Figs. 4–10 show the phase appearance of the brush

border of intestinal epithelium when mounted in a series of fluids of known RI. The border showed an extreme degree of phase-positive contrast in a medium of RI 1.47, and of phase-negative contrast at RI 1.62. The actual change of phase, as will be seen, occurred between RI 1.5521 and RI 1.560.





Text-fig. 3 (*left*). Effect on the RI of the brush border of intestinal cells.
Text-fig. 4 (*right*). Effect of fixation on the RI of the nuclear membrane of intestinal epithelium.

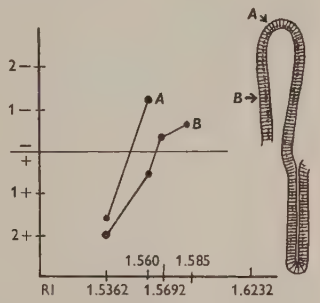
These findings are expressed in graphic form in Text-fig. 2, where phase appearance was plotted against RI. The RI of brush border may be read off from the point at which the curve crosses the ordinate.

Microanatomical results.—By deliberate selection of a mountant producing maximum contrast, an extremely detailed picture could be obtained of any particular structure. The brush border of the intestinal epithelium shows very clearly in Fig. 11; Paneth cell granules, in negative contrast, in Fig. 12; apical vacuoles, brush border and mitochondria of mesonephric tubules in Fig. 13.

The appearance of cells in the zona glomerulosa and fasciculata of the suprenal is shown in Figs. 16 and 17. Intracellular granules of two types may be seen in the glomerulosa. Some are strongly phase-positive and appear almost black; others are phase-negative, appearing white against the grey cytoplasm. In the fasciculata, there are phase-negative intracellular vacuoles containing phase-positive material. Very scanty, minute phase-positive granules were also encountered. The cells of the anterior hypophysis could easily be differentiated into α, β and chromophobe varieties.

Influence of fixative on RI.—The RI of the intestinal epithelial brush border is influenced to a surprising extent by the fixative employed. The effect of different fixatives is shown in Figs. 18–22; the mountant RI was 1.5521. The photographs could be arranged in a series. At one end, the effect of some fixatives (Champy) was to produce a strongly phase-positive border,

while at the opposite end were fixatives producing a phase-negative appearance. After some fixatives (Da Fano, Bouin), the border was phase-neutral, being invisible, or nearly so, in the mountant employed. The effect of other fixatives upon the RI is recorded in Text-fig. 3.



Text-fig. 5. Regional variation of the RI of the epithelial brush border in different parts of a villus. Flemming fix. (Compare figs. 27, 28.)

A similar variation was seen with other structures. Text-fig. 4 shows the effect of fixative upon the RI of the nuclear membrane.

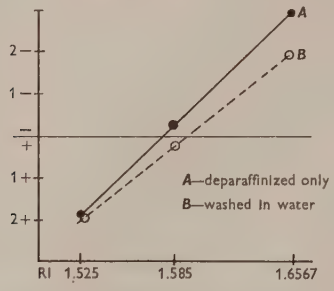
Gradients of RI.—It was found that the RI of intestinal epithelial cells forms an obvious gradient, varying appreciably from the villus tip down the side of the villus to the bottom of the Lieberkühn crypt. When examined (after Helly fixation) in a medium of RI 1.5600, the brush border at the tip was almost phase-neutral (invisible). The apical cytoplasm of the cells was phase-negative (Figs. 23–26).

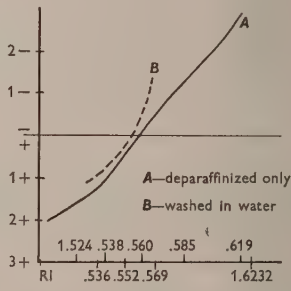
A short distance down the villus, the brush border had become obviously phase-negative; perinuclear Golgi zone and apical cytoplasm were easily visible. At about mid-villus, the brush border was still distinct, but contrasts were much less for the other structures. At the top of the Lieberkühn crypt, all traces of cytoplasm and nuclei had disappeared; all that could be seen were the brush border zone and mucus within goblet cells, both of which were in negative contrast. At the bottom of the crypt, the only structures visible were phase-negative collagen fibres and goblet cells, and the phase-positive granules of Paneth cells.

Regional differences in the RI of the intestinal brush border were seen after other fixatives. With Flemming, for instance (Figs. 27, 28), the apical border was obviously phase-negative, but became phase-positive a short way down the villus f.p. This result is expressed graphically in Text-fig. 5.

Effect of washing.—Experiments were made on tissue fixed with the usual standard watery solutions, and on tissue prepared by freeze-drying and freeze-substitution.

WATERY SOLUTIONS. After being washed in acidified water, the RI of the intestinal epithelial brush border was considerably changed. The appearances after Flemming fixation are shown in Fig. 14. When mounted in a fluid of RI 1.585, the border was perceptibly paler than the background before washing, but after rinsing in acidified water the border became the





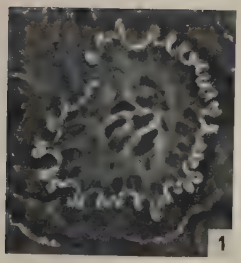
Text-fig. 6 (*left*). Effect of washing in acidified water upon the RI of the intestinal brush border. Flemming fix. (Compare figs. 14, 15.)
Text-fig. 7 (*right*). Effect of washing in water upon the RI of the nuclear membrane (intestinal epithelial cells, Helly fix.).

darker (Fig. 15). The RI of the border, therefore, was raised by rinsing (Text-fig. 6).

Similar experiments showed that the RI of the nuclear membrane also was altered by washing in water (Text-fig. 7). The RI, as will be seen, was in this case lowered.

All photomicrographs were of unstained sections in phase illumination, × 95 oil immersion objective.

Figs. 1–3. Optical differentiation of tissues of the same animal, fixed in the same fluid (Helly) and mounted in the same medium (RI = 1.552). *1.* Artery showing strongly phase-negative elastic membrane. *2.* Mast cells. Note phase-negative granules. *3.* Pancreas. Note phase-positive zymogen granules.
Figs. 4–10. Intestinal epithelial cells of the same villus mounted in media of different RI. From left to right: 1.47, 1.52, 1.55, 1.560, 1.569, 1.58, 1.62. Cat intestine fixed in Helly fluid.
Fig. 11. Intestinal epithelial cells mounted in chloro-benzene. Mouse, Flemming fix.
Fig. 12. Paneth cells mounted in quinoline (RI = 1.62). Mouse, Champy fix.
Fig. 13. Frozen-dried embryonic fowl mesonephros showing mitrochondria. Liquid paraffin (RI = 1.47).
Fig. 14. Brush border of intestinal epithelium. The section was deparaffinized and mounted in aniline (RI = 1.58). Mouse, Flemming fix.
Fig. 15. The same place after washing in acidified water; remounted in the same medium. The brush border now appears phase-positive.
Fig. 16. Mouse suprarenal, zona glomerulosa, mounted in nitrobenzene, showing strongly phase-positive vacuoles in the cells.
Fig. 17. Same section as preceding, zona fasciculata. Note phase-negative vacuoles containing darker material.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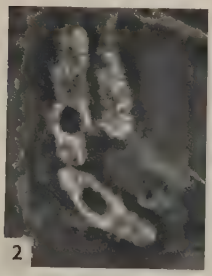
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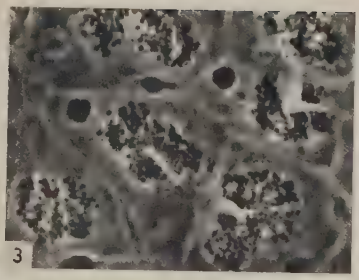
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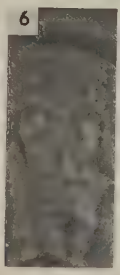
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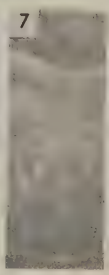
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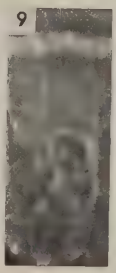
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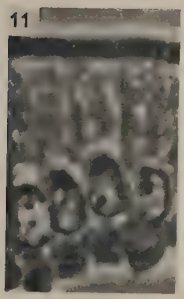
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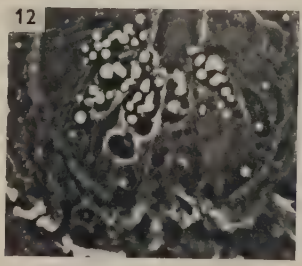
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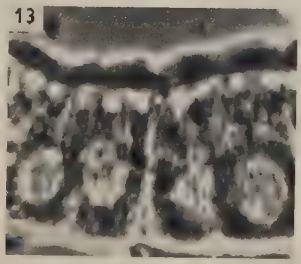
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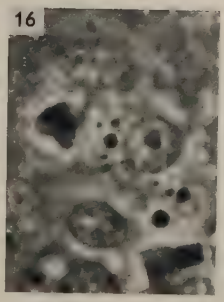
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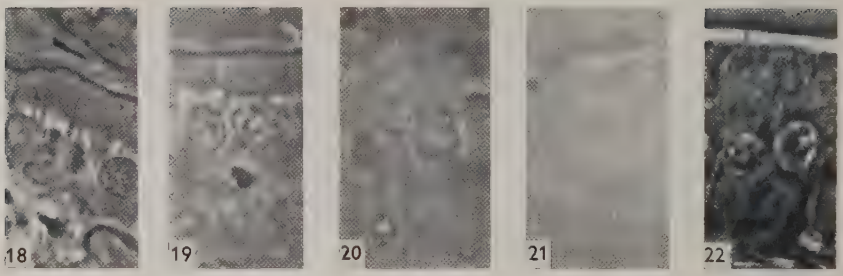


Figs. 18–22. Effect of fixative on the RI of mouse intestinal epithelial cells. Mountant of RI = = 1.552. From left to right: Champy fix., Helly, Da Fano, Bouin, mercuric chloride and formol.

FREEZE-DRYING AND FREEZE-SUBSTITUTION. Contact with water was of course avoided in tissue prepared by these techniques. It was expected therefore that their water-soluble components would remain preserved more or less intact in deparaffinized sections mounted in oily fluids, and that on subsequent washing in water there would be a considerable loss by solution.

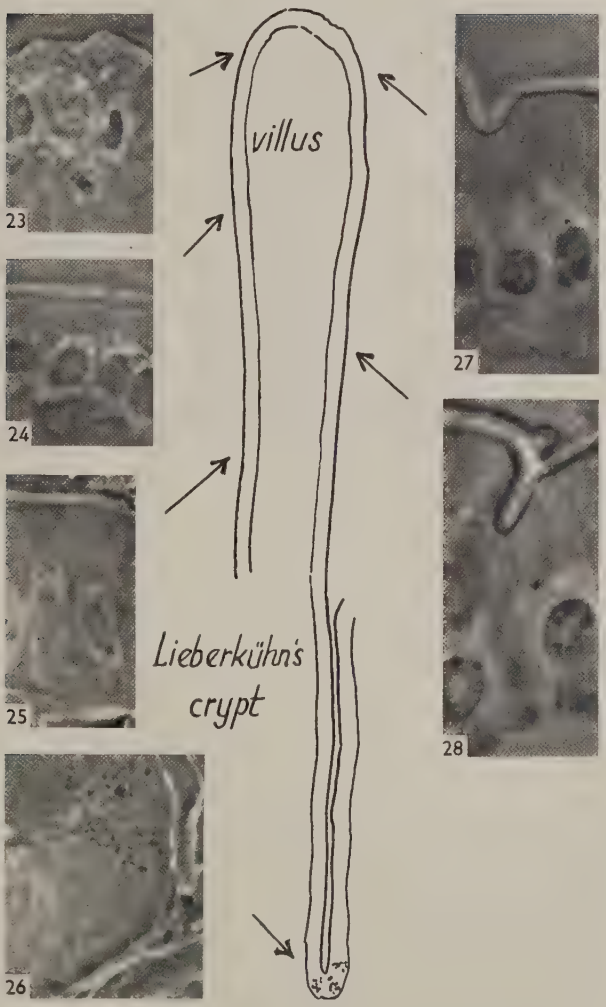
The effect of washing water upon the RI of rat kidney prepared by freeze-substitution is shown in Figs. 29–36 and in Textfig. 8. A noticeable loss of contrast in the peritubular basement membrane and epithelial brush border after washing is illustrated in Figs. 29–36; in Textfig. 8, the full range of observations made in ten mountants is expressed in graphic form. It will be seen that the RI was raised considerably by washing.

DISCUSSION

The general validity of the method for measuring the RI of an object, by examining it in fluids of known RI, seems to be fully established [2–11, 13–15, 19]. The question arises as to whether the results described above were affected by subjective or technical factors.

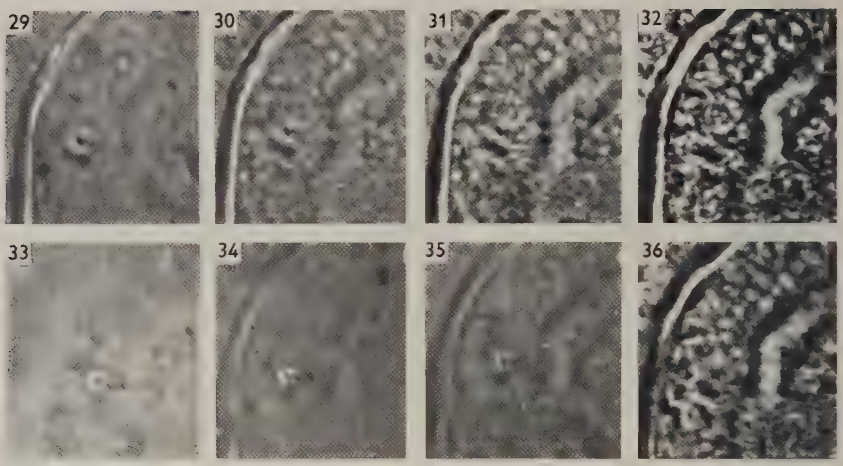
In theory, subjective factors may have influenced the quantitative assessment of degree of phase contrast. In practice, however, no attempt was made to strain discrimination, and when the actual photographs (Figs. 4–10) are compared with the inferences drawn from them (Textfig. 2), the possibility of serious misinterpretation seems remote.

Theoretically, technical factors might have influenced the results in four ways. First, there is the question of permeation of section by mountant. This, as stated above, caused initial difficulty and could produce misleading results, but, once recognized, the difficulty was easily resolved. Second, the



Figs. 23–26. Optical differentiation of intestinal epithelial cells from different parts of a villus and Lieberkühn's crypt. Mouse, Helly fixation, bromo-benzene mountant.
Figs. 27, 28. Similar differentiation of the brush border of intestinal epithelium after Flemming fix.

actual values of RI might have been affected by dilution or degradation of the mounting fluids; this remains a possibility, as no means of checking the indices of refraction of the samples at the conclusion of the experiments were available. Third, as with all phase-contrast observations, there was the possibility of halo effects vitiating the results. However, this can be excluded

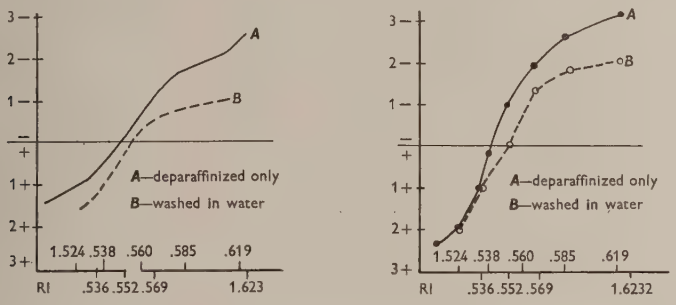


Figs. 29 32. Rat kidney (freeze-substitution). Same field mounted in media as follows. From left to right: RI 1.52, 1.560, 1.569, 1.61.
Figs. 33 36. Same field after section had been washed in water and remounted in the same media.

in the present work since haloes were easily recognizable and were distributed well outside the structures under observation. Fourth, it is theoretically possible that, in spite of attempts to maintain a constant section thickness, some individual variation nevertheless occurred. This would affect the relative light transmission of different sections, since phase change is a product of both RI and thickness [8]. However, the possibility of serious error seems slight, as the comparative observations were carried out on different tissues blocked together and cut at the same stroke of the microtome. It should be pointed out that the use of mountants whose RI differed greatly from that of the section might well give misleading results: under these circumstances, the transparency of the section might fall very much below that of the mountant so that amplitude as well as phase differences would be introduced. Such mountants were deliberately avoided in the present experiments.

It is interesting that the RI of the intestinal epithelial cells and their brush borders forms a demonstrable gradient from the tips of the villi, along their sides and down to the crypts. This agrees in general with observations on other attributes of this epithelium, for instance with the distribution of alkaline glycerophosphatase [17, 20]. It may also be a reflection of differences in the cells as they travel up the length of the villus [18].

As the photographs of the suprarenal show, inclusions in the cells of the zona glomerulosa are clearly divisible into two groups, differing widely in their refractive index. By ordinary staining methods, all the inclusions look the same. This suggests that structures giving the same staining reactions may possess widely different refractive indices, a possibility which is now being investigated further. If such proves to be the case, an entirely fresh



Text-fig. 8. Effect of washing in water upon the RI of rat kidney prepared by freeze-substitution. A, Brush border of proximal convoluted tubules: B, Basement membrane.

means of histological investigation may be available. It may also be possible to use measurements of RI in quantitative histochemistry. The method can detect changes in the RI of sections washed in water, and therefore it should be possible to detect changes produced by reagents such as periodic acid, ribonuclease, decalcifying agents and the like [12].

Variation in the RI of the brush border of the intestinal epithelial cells after treatment with different fixatives does not seem to have been reported before. Crossmon [11], for instance, worked only with formol-fixed material. It was probably due to multiple causes, the precise chemical or physical nature of which remains obscure. The fact that the RI was high after fixation in Champy's fluid suggests that some sort of osmium-tissue complex was produced; Bahr [1] has recently brought forward evidence that such compounds are formed. Whatever the explanation may be, certainly this fixative acted as the best "stain" for the phase-contrast microscopy of unstained sections.

RI changes in sections rinsed in water presumably reflect either removal of water-soluble substances or hydration of others, or express the sum of both processes. It seems logical to suppose that the RI of the nuclear membrane was lowered after rinsing in water (Text-fig. 7) because water-soluble

material was extracted. There is no doubt that water-soluble materials can be removed in quantity from sections in this way, as is shown by the following simple experiment. A section of tissue prepared by freeze-substitution in alcohol was covered with a droplet of redistilled water; the latter was removed with a micropipette, delivered on to a slide, and allowed to evaporate. A surprising wealth of crystals was deposited in comparison with controls. On the other hand, the RI of the brush border and peritubular basement membrane of kidney prepared by freeze-substitution (Text-fig. 8) and of the intestinal epithelial brush border (Text-fig. 6) rose quite considerably on washing. This seems to suggest that water was incorporated into some compounds occurring at these sites. It is interesting to speculate on the possible implication of mucopolysaccharides which are known to be present in high concentration in brush borders and renal peritubular basement membrane. It may be that water is lost on dehydrating the tissue, and reincorporated on exposing a section to water.

SUMMARY

1. A method for measuring the refractive index of sectioned tissue is described. It entails phase-contrast observation of sections mounted in fluids of known refractive index.

2. The refractive indices of structures such as intestinal and renal epithelial brush borders, nuclei and cytoplasm were investigated.

3. The epithelial cells of the small intestine and their brush borders showed a gradient of refractive index passing from villus tip to base of crypt.

4. The cytoplasmic inclusions in the zona glomerulosa of the suprarenal, which have the same staining reactions, comprised two groups with widely different refractive indices.

5. The refractive index of fixed structures varied according to the fixative employed. For instance, with Champy's fluid, it was high; with mercuric-chloride formol, it was low.

6. The refractive index of tissue sections was altered by washing them in water. The refractive index of the nuclear membrane fell, but that of brush borders and peritubular basement membrane rose.

7. These findings are discussed and tentative explanations suggested.

We are greatly indebted to Dr. R. Barer for criticism of this work.

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THE EFFECT OF X-IRRADIATION ON THE LENGTH OF THE MITOTIC CYCLE IN *VICIA FABA* ROOTS

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The reduction in the growth-rate of *Vicia faba* roots after irradiation has been variously used in radiobiological experiments as a measure of radiation damage. The processes intervening between irradiation and the observation of reduced growth-rate have not been established. Gray and Scholes [1] have shown that the diminished growth-rate results partly from a reduction in the length attained by elongating cells and in the size of the meristem. They suggest that, for the rest, the reduced growth-rate is due to the combined effect of cell death and the lengthening of the mitotic cycle. In order to show the relative importance of these last two factors it is necessary to evaluate one independently of the other.

In this paper the length of the mitotic cycle after irradiation is assessed by the incidence of micronuclei after a second irradiation.

When a chromosome is broken by irradiation and the broken ends do not rejoin, the acentric fragment takes no part in the metaphase and anaphase movements of mitosis and is usually excluded from the daughter nuclei in telophase. The fragment becomes spherical and forms a micronucleus enclosed in one of the daughter cells (Fig. 1). The period between irradiation and the occurrence of the maximum number of cells containing micronuclei is directly related to the length of the mitotic cycle.

METHOD

Vicia faba seeds (Sutton's Prolific Longpod) were germinated and grown in aerated tap water at 19°C as described by Gray and Scholes [1]. When the roots were 10–16 cm long, three groups, matched for root-length, were exposed to 140 or 50 r of X-rays. One group, which received 140 r, was irradiated again 3 days later with 50 r. All irradiations were made with 190 kV and 4 mA X-rays; $1^1/_2$ mm Cu and 1 mm Al filters were used.

At various intervals after irradiation the roots were fixed in acetic acid–ethanol mixture (1:3 v/v) (Table I); they were stained *in toto* by a modification of the Feulgen technique, and the meristem was cut off and macerated for the preparation of cell-suspensions as described previously [2]. Eight to ten roots were used to make

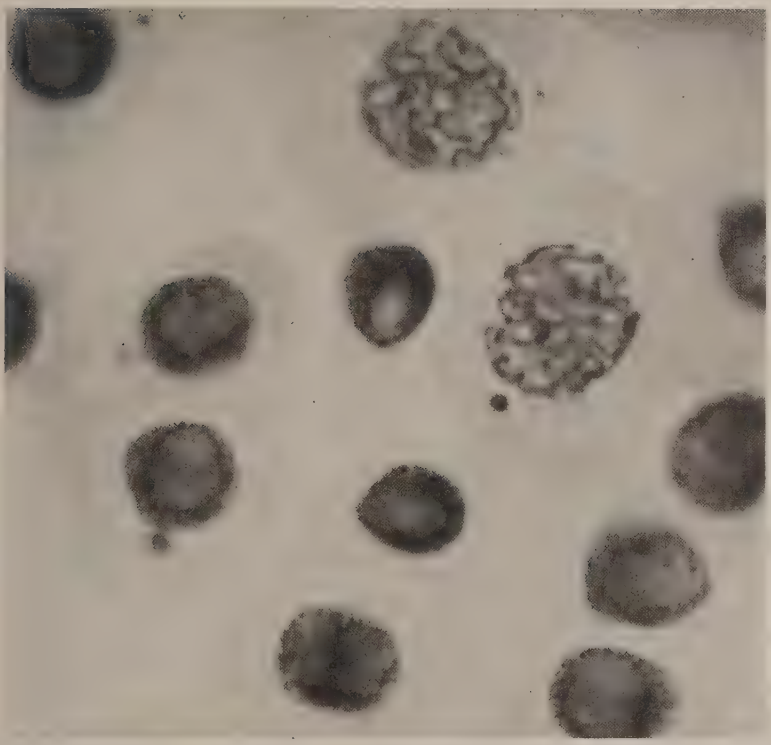

Fig. 1. Photomicrograph. Meristematic cells of *Vicia faba* roots after irradiation, showing cells containing micronuclei. *Top centre:* cell in prophase contains a micronucleus. *Bottom centre:* two interphase cells contain micronuclei which are slightly out of focus.

each cell-suspension, and two samples from the suspension were taken for microscopic examination.

TABLE I

Group	1st irradiation	2nd irradiation (3 days after 1st)	Time of fixing after last irradiation
I	140 r X-rays	None	12 hrs., 24 hrs., 36 hrs., 48 hrs. 3 days, 4 days, 6 days, 8 days, 10 days
II	50 r X-rays	None	12 hrs., 24 hrs., 36 hrs., 48 hrs. 4 days
III	140 r X-rays	50 r X-rays	12 hrs., 24 hrs., 36 hrs., 48 hrs. 4 days

RESULTS

The percentage of cells containing micronuclei after a single dose of 140 r reaches a maximum between 48 and 60 hours. It then falls, and 8 to 10 days after the irradiation, has reached negligible values (Fig. 2 and Table II). After a single dose of 50 r X-rays the maximum is reached at 36 hours (Fig. 2, Table II).

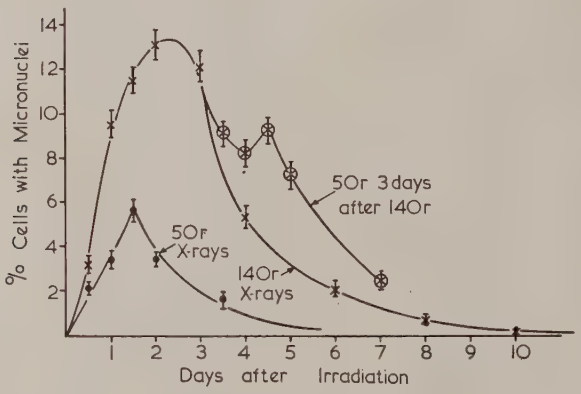

Fig. 2. The percentage of cells showing micronuclei in the meristem of *Vicia faba* root tips at various times after irradiation.

A normal mitotic cycle of *Vicia faba* roots has been estimated to have a mean length of 24–30 hours [1, 3]. Howard and Pelc [3] have shown that some cells in the first mitotic cycle after irradiation are delayed in entering division, and others in beginning DNA synthesis. Thus, the mean length of the first mitotic cycle after irradiation is increased. After X-irradiation, the occurrence of the maximum number of cells containing micronuclei at approximately 48 hours after 140 r and at 36 hours after 50 r probably reflects this delay in the first mitotic cycle.

The length of the mitotic cycle three days after 140 r of X-irradiation is obtained by determining the time of appearance of the maximum number of cells with micronuclei after a second irradiation of 50 r given at this stage. This maximum occurs 36 hours after the second irradiation, as does the maximum in roots given only a single dose of 50 r X-rays (Fig. 2, Table II). This suggests that any delay in the mitotic cycle resulting from an X-irradiation by 140 r has disappeared within three days.

Table II

Group	Time of fixation after last irradiation	Number of beans	Total cells counted	Per cent of cells with micronuclei	Per cent of cells in mitosis
140 r only	12 hours	9	2,248	3.2±0.4	7.8±0.6
	24 hours	9	2,367	9.5±0.6	6.3±0.5
	36 hours	9	2,489	11.5±0.6	8.4±0.6
	48 hours	8	2,562	13.1±0.7	8.9±0.6
	3 days	9	2,457	12.1±0.7	7.0±0.5
	4 days	10	2,254	5.3±0.5	6.4±0.5
	6 days	8	2,207	2.1±0.3	7.3±0.6
	8 days	8	2,331	0.7±0.2	9.3±0.6
	10 days	8	1,085	0.2±0.1	7.7±0.8
140 r X-rays	12 hours	8	2,378	9.1±0.6	7.2±0.6
followed after	24 hours	8	2,300	8.2±0.6	5.4±0.5
3 days by	36 hours	9	2,392	9.2±0.6	7.6±0.6
50 r X-rays	48 hours	9	2,278	7.2±0.6	5.2±0.5
	4 days	9	2,183	2.4±0.3	6.1±0.5
50 r only	12 hours	8	2,376	2.1±0.3	13.7±0.8
	24 hours	8	2,274	3.4±0.4	8.8±0.6
	36 hours	9	2,347	5.6±0.5	9.4±0.6
	48 hours	9	3,424	3.4±0.3	9.1±0.5
	3½ days	8	2,262	1.6±0.3	8.4±0.6
Controls	—	8	2,196	0	8.9±0.6

DISCUSSION

A micronucleus is produced when a cell with an acentric fragment completes division and the fragment is excluded from both daughter nuclei. Provided that the micronuclei remain visible for at least one mitotic cycle, the number of cells containing micronuclei will increase until all irradiated cells have completed their first division, and as cells go through their second division the proportion of cells containing micronuclei will diminish. Gray and Scholes [1] showed that the proportion of cells containing micronuclei after the first division declines more rapidly than would be expected if the decrease were due only to the increasing total of cells resulting from further division; the decrease must result, at least in part, from the disappearance

of the micronuclei themselves. Some micronuclei, however, survive at least until the initial stages of the second division, for cells are seen in prophase and metaphase with micronuclei which have been produced not later than the previous division (Fig. 1). It seems unlikely, therefore, that the disappearance of micronuclei affects the time when the maximum number of cells containing micronuclei occurs. There is evidence that there is a period

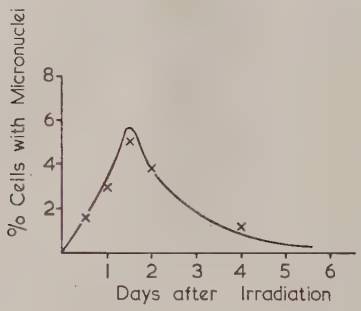

Fig. 3. The curve shows the percentage of cells with micronuclei after a single dose of 50 r X-irradiation and is redrawn from Fig. 2. Cross: Percentage of cells which contain micronuclei attributable to a dose of 50 r X-rays given 3 days after 140 r X-rays.

in the mitotic cycle during which irradiation is particularly liable to produce chromosome aberrations [4]. Consequently, there will be an increase in the rate of production of micronuclei at a time after irradiation when cells that were in this critical state during the irradiation have passed the subsequent division. If, however, injury causing chromosome-breaks is produced at all stages of the mitotic cycle, the maximum number of cells containing micronuclei will occur at the end of the subsequent mitotic cycle.

If after the first irradiation a large proportion of cells do not divide again, but remain in the meristem until differentiation or death, then after a second irradiation they will not produce micronuclei, and the maximum number of cells containing micronuclei at this stage will be lower than that produced by a single dose of irradiation. The number of micronuclei produced by the second irradiation is given by subtracting from the observed values the number of micronuclei which remain from the first irradiation. The number so obtained is found to be the same as that seen after a single dose of 50 r X-rays (Fig. 3).

It can therefore be concluded that the length of the mitotic cycle of *Vicia faba* roots three days after 140 r X-irradiation is the same as that of unirradiated roots. Thus, the length of the mitotic cycle three days after irradiation does not contribute to the reduced growth-rate observed at this time.

SUMMARY

1. The length of the mitotic cycle after an irradiation is assessed by the interval elapsing between a second exposure and the highest count of cells containing micronuclei.

2. It is found that, three days after bean roots have been irradiated with 140 r X-rays, the length of the mitotic cycle is the same as that of unirradiated roots.

I am indebted to Dr. L. H. Gray, who suggested this work, and to Dr. A. Howard, who gave advice and encouragement.

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CYTOCHEMICAL STUDIES ON THE MITOTIC SPINDLE AND THE PHRAGMOPLAST OF PLANT CELLS

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In order to reveal the mechanism of cell division, it is necessary to understand its chemical aspects, viz., to recognize cell division as a series of chemical or physicochemical processes within the cell. One way of approach may be to inquire what is the chemical composition of the mitotic organelles, especially that of the mitotic spindle and the phragmoplast. As far as the present authors know, there have been only a few reports on the chemical composition of the spindle and the phragmoplast. The chief object of previous works was to describe the chemistry of resting nuclei or the chromosomes; some notes on the so-called achromatic figure have been written merely as additions. Earlier publications on pentosenucleic acid in the achromatic figure of both plant and animal cells are summarized in Table I, relatively recent studies about proteins are listed in Table II.

The present authors investigated the mitotic spindle and the phragmoplast of fixed plant cells by means of staining methods, and proved that both contained pentosenucleic acid as a chemical component. A test was carried out also on polysaccharides in the mitotic spindle and the phragmoplast. Monné and Slautterback [15] and Stich [28] published reports on polysaccharides in the mitotic organelles of animal cells, but so far there seems to have been no publication concerning the occurrence or distribution of polysaccharides in the spindle and in the phragmoplast of plant cell.

MATERIALS AND METHODS

For somatic cells, we used the root tip cells of *Allium cepa*, *Allium scorodoprasm* var. *viviparum*, *Lilium japonicum*, *Tradescantia canaliculata*, *Zea mays*, *Pisum sativum*, and *Vigna sesquipedalis* and also the stamen-hair cells of *Tradescantia*. We used the pollen mother cells of *Lilium* and of *Tradescantia*.

In some cases we experimented with the centrifuge. Centrifugal forces of 500–

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TABLE I

List of the previous studies about pentosenucleic acid (PNA) in the mitotic spindle.

Materials	Methods	PNA in mitotic spindle		Investigators
		Poleward region	Interzonal region	
PLANT CELLS				
Root tip cell of *Vicia*	Fixed material Methyl green–pyronin	(stainable by pyronin)		Shinke & Shigenaga [25]
Root tip cell of *Allium*	Fixed material Ultraviolet absorption	Absorption + (weak)		Ris [21]
Root tip and pollen mother cells of *Allium, Lilium Tradescantia* and *Vicia*	Fixed material Safranin	PNA +		Kaufmann *et al.* [9, 10]
Stamen-hair cell of *Tradescantia*	Living material Ultraviolet absorption	Absorption +		Aimi & Yamaha [1]
Proembryo of *Pinus*	Fixed material Toluidine blue, thionin, methyl green–pyronin	PNA +	PNA +	Shimamura [24]
ANIMAL CELLS				
Egg of frog	Fixed material Methyl green–pyronin	(stainable by pyronin)		Brachet [2]
Eggs of urodelae, spermatocyte of grasshopper, etc.	Fixed material Methyl green–pyronin	PNA + (Intense in eggs, slight in spermatocytes)		Brachet [3] (pp. 235–236)
Egg of sea urchin	Living material Ultraviolet absorption	Absorption —		Harvey & Lavin [5]
Egg of *Ascaris*	Fixed and stained material	PNA +		Pasteels [20]
Spermatocyte of grasshopper	Fixed material Safranin	PNA +		Kaufmann *et al.* [9, 10]
Eggs of *Cyclops*, etc.	Fixed material Toluidine blue	PNA +		Stich [27, 29, 30]
Cultured fibroblast of chick embryo etc.	Fixed material May-Grunwald and Giemsa	PNA —	PNA + (intense)	Jacobson & Webb [8]
Cultured fibroblast of chick embryo	Living material Ultraviolet absorption	Absorption — (or little)	Absorption + (intense)	Davies [4]
Egg of *Solenobia*	Fixed material Toluidine blue, methyl green–pyronin	PNA +	PNA + (less than in poleward region)	Ris & Kleinfeld [22]

TABLE II

List of the previous studies about proteins in the mitotic spindle.

Materials	Methods of staining	Proteins in mitotic spindle		Investigators
		Poleward region	Interzonal region	
PLANT CELLS				
Root tip cells of *Vicia*, *Zea*, etc. Pollen mother cell of *Lilium*	Millon's reaction	+	(Phragmoplast) + (intense)	Shinke & Shigenaga [25]
Pollen mother cell of *Lilium*	Bromophenol blue–mercury	+ (less than cytoplasm)		Mazia *et al.* [13]
Proembryo of *Pinus*	Ninhydrine–Schiff, bromophenol blue–mercury	+	+	Shimamura [24]
ANIMAL CELLS				
Egg of sea urchin	Isolation of mitotic apparatus in quantity	A protein of single type Isoelectric pt. 6 Particle wt. 45,000		Mazia & Dan [12]
Egg of *Ascaris*	Bromophenol blue–mercury	+ (intense)		Mazia *et al.* [13]
Egg of *Cyclops*, etc.	Fast green, acid fuchsin	+ (basic proteins)		Stich [29, 30]

700 *g* or 1050–1300*g* were applied to the floral buds of *Lilium* for 15 minutes before fixation.

Root tips, floral buds (*Tradescantia*) and anthers (*Lilium*) were fixed for about 10 hours in Carnoy's (without chloroform) or Telyesniczky's fixatives. Carnoy's fixative can satisfactorily preserve nucleic acids and proteins, causing, however, some deformation of cellular structures. On the other hand, Telyesniczky's fixative has little influence on cellular structures, and the coloration of preparations fixed in this fluid appears to be weaker than those fixed in Carnoy's fixative.

Jacobson and Webb [8] employed 100 per cent methanol for fixation of the chick fibroblasts cultured *in vitro* with good results. But in plant materials 100 per cent methanol causes a destructive shrinkage of cells, sometimes with deformation of tissues.

Fixed materials were then dehydrated through a graded series of ethanol and *n*-butanol in routine manner and embedded in Tissuemat (Eimer & Amend, m.p. 52–54°C), care being taken to avoid exposure to temperatures above 60°C, lest nucleic acids be depolymerized.

Sections of a thickness of 10 or 15 μ were prepared. After Tissuemat was removed from the sections by xylene, they were stained with the methods described below.

Staining of nucleic acids.—In order to detect nucleic acids, we applied differential staining by toluidine blue, thionin and a combination of methyl green and pyronin.

One per cent solution of toluidine blue O (Fisher) and one per cent solution of thionin (Fisher) were prepared. Sectioned materials were stained for 30 minutes, then rinsed in distilled water for two minutes, and put into 80 per cent ethanol and left for 10–20 hours for differentiation. These dyes stain nucleic acids differentially by metachromasis.

Methyl green (Merck) was purified with chloroform, and pyronin B (Grübler) with chloroform, *n*-butanol and ether by the procedures recommended by Sibatani [26]. A solution was prepared of final concentrations with 0.3 per cent methyl green, 0.6 per cent pyronin, and 0.14 *M* acetate buffer (pH 4.4). Sections were stained for 30 minutes, rinsed in distilled water for not more than two minutes and differentiated in 100 per cent ethanol for one to two hours.

After differentiation the sections were dried in air, cleared in xylene and mounted in Canada balsam.

To determine whether pentosenucleic acid was present, before staining hydrolysis tests were made on some preparations of root tip cells of all the plants studied and on the pollen mother cell of *Lilium*. As hydrolytic reagents, we applied pentosenuclease, perchloric acid ($HClO_4$), and hydrochloric acid (HCl). Pentosenuclease (Worthington Biochemical Laboratory) was used in a solution of 0.4 mg/ml buffered at pH 7.5 with borate, and hydrolysis was carried out at 47°C for one or one and a half hours. The hydrolysis test with 10 per cent $HClO_4$ was done at 3–4°C for 18 hours and that with 1 *N* HCl was done at 60°C for five or eight minutes. As controls, sections were prepared from the same root tip or anther under the same conditions but without the hydrolytic reagent.

To ascertain whether the mitotic spindle and the phragmoplast increased the stainability with acidic dyes, we stained them with fast green or orange G after hydrolysis of pentosenucleic acid. After hydrolysis some of the preparations of the pollen mother cell of *Lilium* and the root tip cells of *Allium*, *Tradescantia* and *Zea* were stained with one per cent fast green for 30 minutes or with one per cent orange G (Grübler) for five minutes.

Staining of proteins.—For staining proteins, we employed the bromophenol blue–mercury method of Mazia *et al.* [13], ninhydrine-Schiff and alloxan-Schiff methods of Yasuma and Ichikawa [33].

The ninhydrine-Schiff and alloxan-Schiff methods were carried out as follows. Sections were put in 0.5 per cent solution of ninhydrine in ethanol or in one per cent solution of alloxan in ethanol for six or more hours at 37°C, then rinsed in running water for a few minutes and stained in Schiff's reagent for two or more hours. Schiff's reagent here employed was composed of 0.5 per cent basic fuchsin (Merck), 0.1 *N* HCl, and 0.5 per cent potassium pyrosulfite ($K_2S_2O_5$). After staining the preparations were washed three times in 0.5 per cent $K_2S_2O_5$, then in running water, dehydrated and cleared through a graded series of ethanol and xylene, and mounted in Canada balsam.

Staining of polysaccharides.—Lillie's method [11] for staining polysaccharides was

applied. Sections were oxidized in potassium periodate (KIO_4) solution (0.8 g of KIO_4 and 0.3 ml of 70 per cent nitric acid in 100 ml of water) for five minutes, then after a wash in distilled water they were stained in Schiff's reagent for 30 minutes. The subsequent procedures were the same as in staining of proteins.

OBSERVATIONS

The Pentosenucleic Acid

Late prophase.—In the root tip cells, so-called polar caps show a fibrous texture after fixation and this texture runs in the same direction as the mitotic axis. The polar caps are stained a bluish violet by toluidine blue, purple by thionin and red by methyl green pyronin, thus proving the presence of pentosenucleic acid. In the nuclear cavity the chromosomes are already formed and are stained blue by either toluidine blue or thionin and a bluish green by methyl green–pyronin, manifesting the presence of desoxypentosenucleic acid. No other stained material, however, is recognized in the nuclear cavity.

In the stamen-hair cell of *Tradescantia* the same results are obtained as in the polar caps of the root tip cells.

In the pollen mother cell of *Lilium* in prometaphase of the first meiotic division, the chromosomes assemble in the center of the cell. Surrounding the chromosome group, numerous fibers are observed with no definite polarity. This fibrous area is stained an intense bluish violet by toluidine blue, a deep purple by thionin and a rich red by methyl green–pyronin, showing a plentiful accumulation of pentosenucleic acid in this area similar to that in the polar caps of somatic cells.

Metaphase.—A distinct fibrous structure is produced by fixation in the mitotic spindle in metaphase. The spindle is stained an intense bluish violet by toluidine blue, a deep purple by thionin and a rich red by methyl green–pyronin. These colors indicate a large accumulation of pentosenucleic acid. The spindle fibers themselves are conspicuously stained, while the cytoplasm, also containing pentosenucleic acid, is less conspicuous, as shown in Figs. 1*a*, 2*a* and 6.

This staining reaction is common to the preparations fixed in both Carnoy's and Telyesniczky's fixatives and for both somatic cells and pollen mother cells.

Anaphase.—In anaphase as well as in metaphase, the conical poleward regions of the mitotic spindle exhibit a distinct fibrous structure converging at the poles. These regions are stained a bluish violet by toluidine blue,

purple by thionin and red by methyl green–pyronin, although the colors are somewhat weaker than in metaphase.

On the other hand, the interzonal region between the separated daughter chromosome groups shows quite different features. This region is apt to be damaged by fixatives or other agents during the course of preparation and exhibits various kinds of artefact. In some cases a fibrous structure, apparently looser than that in the poleward regions, is produced, which might suggest the presence of so-called connecting fibers ("Verbindungsfasern") (Fig. 5). In other cells, however, granules and irregular networks are observed in this region, making it hardly distinguishable from the cytoplasmic area (Fig. 4). Moreover, sometimes the cellular material of this region has almost disappeared, leaving only a little amount of debris, whereas the poleward regions are fixed in a finely fibrous condition; this effect occurs more frequently in Carnoy-fixed preparations than in those fixed with Telyesniczky fluid. Even among neighboring cells in the same section, the interzonal region is fixed in various ways.

In whatever condition the interzonal region may be fixed, the fibers, the granules, the networks, and the debris show the same intensity of staining reaction for pentosenucleic acid as in the surrounding cytoplasm (Figs. 4 and 5).

Thus, the interzonal region is quite different from the poleward regions not only in its structure after fixation but also in its staining reaction for pentosenucleic acid.

Where the pollen mother cell of *Lilium* was centrifuged in anaphase and the mitotic organelles were shifted and deformed, as in experiments by Shimamura [23], no decisive result has yet been obtained on the structure of the interzonal region.

Telophase.—In telophase, the most conspicuous mitotic organelle is the phragmoplast.

In the root tip cells and in stamen-hair cells the phragmoplast becomes distinct as a spherical or a barrel-shaped body after the daughter chromosomes have gone to the poles and have strongly contracted ("tassement polaire"). The phragmoplast shows a very distinct fibrous structure running parallel to the mitotic axis, and assumes an intense coloration, viz., a bluish violet with toluidine blue, purple (or a pink-purple in some preparations) with thionin and red with methyl green-pyronin, demonstrating an abundance of pentosenucleic acid. The intensity of colors in the phragmoplast is more impressive than in the mitotic spindle in metaphase (Figs. 1 *a* and 7).

It is of interest to note that the cell plate region appears as a colorless line, indicating the absence of pentosenucleic acid.

During the process of cell division, the phragmoplast becomes flattened and the staining reaction to pentosenucleic acid decreases in the central region; this phenomenon extends centrifugally towards the side walls so that the stained areas are observed only near the walls (Fig. 8). A densely fibrous structure is seen only in the areas showing intense coloration.

In the pollen mother cells (*Lilium* and *Tradescantia*) the phragmoplast and the cell plate region show the same features as in the somatic cells described above.

Hydrolysis tests.—When the cells are treated with any one of the following reagents: pentosenuclease, $HClO_4$, and HCl and are then stained, the polar caps, the mitotic spindle and the phragmoplast have no staining reaction for pentosenucleic acid. Several examples are given.

Fig. 1*a* shows the root tip cells of *Allium* stained by toluidine blue; the metaphase spindle and the phragmoplast are colored more deeply than the cytoplasm. On the contrary, the cells shown in Fig. 1*b*, which are taken from the same root tip as those of Fig. 1*a* but were treated with pentosenuclease before staining, exhibit no staining reaction in the spindle, in the phragmoplast or in the cytoplasm, while the resting nuclei and the chromosomes stain normally. Figs. 2*a* and 3*a* show the pollen mother cells of *Lilium* stained by toluidine blue. Coloration in the spindle, the phragmoplast and the cytoplasm does not occur in the preparations treated with pentosenuclease, as shown in Figs. 2*b* and 3*b*.

From these facts it is clear that pentosenucleic acid exists in the mitotic spindle and in the phragmoplast.

The Proteins

Bromophenol blue–mercury, ninhydrine-Schiff, and alloxan-Schiff methods give almost the same results for the staining reaction for proteins in the cell.

In the resting cell the most stainable organelle is the nucleolus and in the dividing cell the chromosomes are the most stainable.

It is interesting to note that the intensity of the staining reaction for proteins in the spindle and in the phragmoplast are of the same degree as the staining reaction in the cytoplasm.

By any one of the above-mentioned three methods for staining proteins, the polar caps, the metaphase spindle and the phragmoplast are beautifully demonstrated in a fibrous state. The cell plate appears to be a transverse

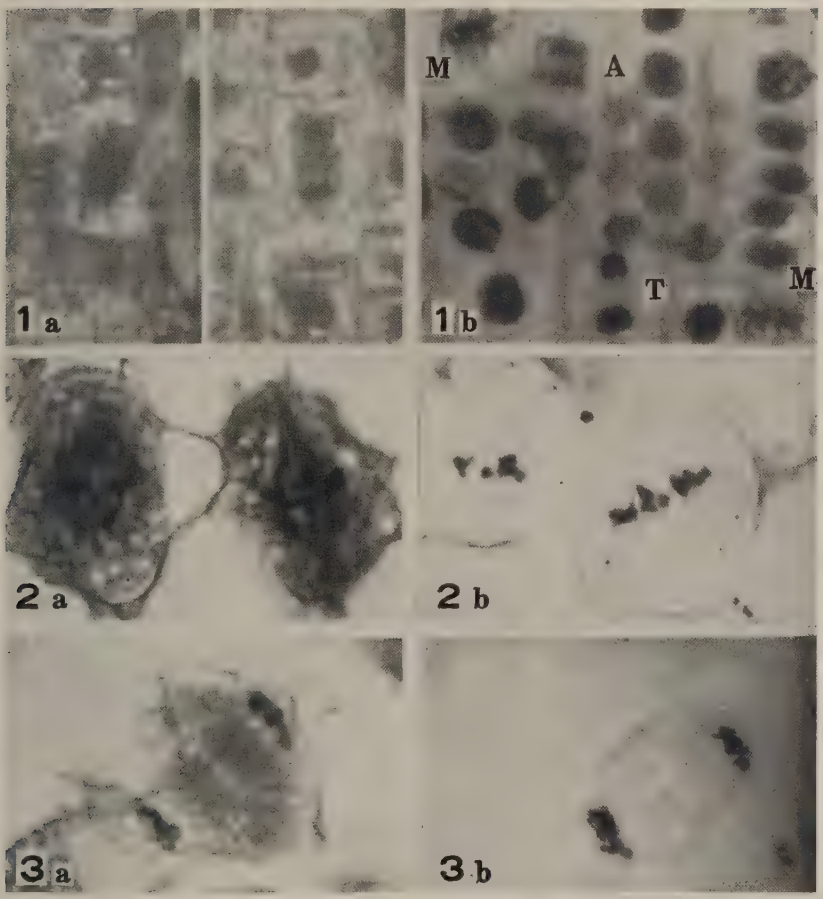

Fig. 1*a*. Root tip cells of *Allium* in metaphase and in telophase. Stained by toluidine blue. × 600.
Fig. 1*b*. The same as in Fig. 1*a*, but treated with PNase before staining. *M*, Metaphase. *A*, Anaphase. *T*, Telophase.
Fig. 2*a*. Pollen mother cells of *Lilium* in metaphase. Stained by toluidine blue. × 600.
Fig. 2*b*. The same as in Fig. 2*a*, but treated with PNase before staining.
Fig. 3*a*. Pollen mother cell of *Lilium* in telophase. Stained by toluidine blue. × 600.
Fig. 3*b*. The same as in Fig. 3*a*, but treated with PNase before staining.

row of small granules. In spite of morphological distinctness, however, the contrast in color intensity between the cellular structures in photographs is unexpectedly too flat to record the details by photography.

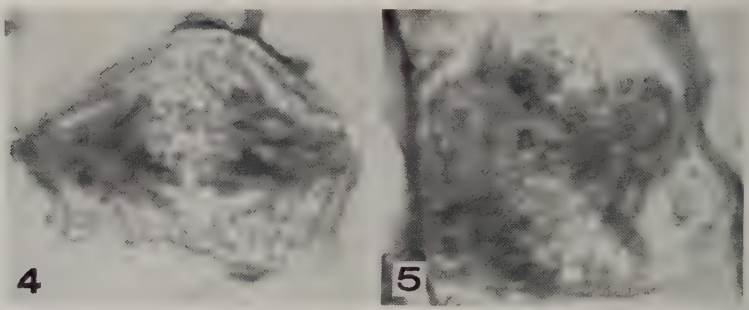

Fig. 4. Pollen mother cell of *Lilium* in anaphase. Stained by thionin. × 1200.
Fig. 5. Pollen mother cell of *Lilium* in anaphase. Centrifuged before fixation, stained by thionin. × 1200.

The Relation between the Pentosenucleic Acid and the Proteins in the Mitotic Spindle and the Phragmoplast

A comparison of the preparations stained by either fast green or orange G after hydrolysis of pentosenucleic acid and the preparations stained by these dyes without hydrolysis reveals that the stainability of cytoplasm, the mitotic spindle and the phragmoplast to these dyes is greater in hydrolysed preparations than in preparations without hydrolysis. The most conspicuous increase in coloration is found in the mitotic spindle and the phragmoplast. Cytoplasm also increases in stainability, but not as distinctly. The chromosomes are the most deeply stained elements in the cell in preparations both with and without hydrolysis of pentosenucleic acid, and it is difficult to decide whether any change in color intensity in the chromosomes is brought about by hydrolysis.

The Polysaccharides

When stained by Lillie's method, the resting nuclei and the chromosomes merely take a very faint pink, almost colorless, except the chromosomes in early telophase which are stained red. In root tip cells the ground cytoplasm

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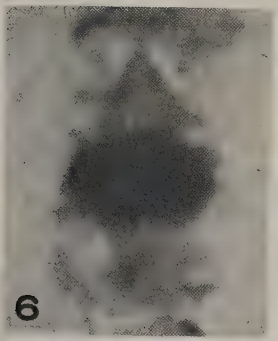
Figs. 6 8. Stamen-hair cells of *Tradescantia*. Stained by methyl green-pyronin. × 1500. Fig. 6, metaphase; Fig. 7, telophase; Fig. 8, late telophase.
Fig. 9. Pollen mother cell of *Lilium* in metaphase. Stained with Lillie's method. The chromosomes in the equatorial region are colorless, while the spindle fibers are stained. × 1200.
Fig. 10. Pollen mother cell of *Lilium* in telophase. Stained with Lillie's method. The cell plate is intensely stained, but the phragmoplast is not. The daughter nuclei are stained. × 1200.
Fig. 11. Root tip cell of *Lilium* in metaphase. Stained with Lillie's method. The chromosomes and the spindle are not stained. Dark dots are starch grains. × 1200.
Fig. 12. Root tip cell of *Lilium* in telophase. Stained with Lillie's method. × 1200.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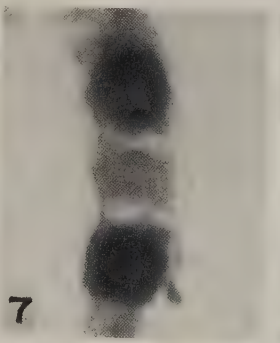
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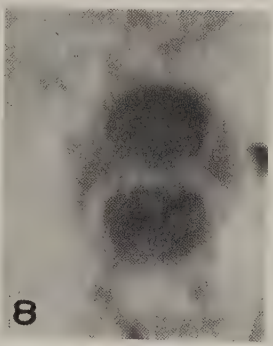
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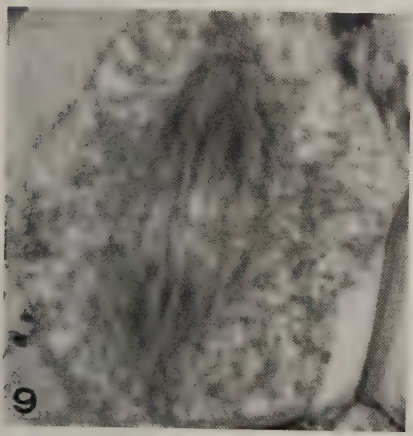
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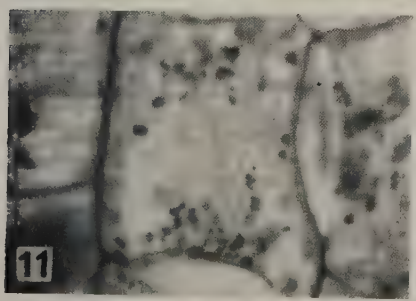
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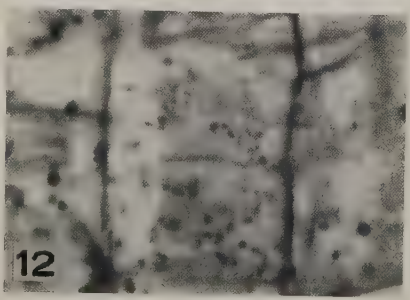
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itself is not stained and the starch grains are colored an intense red-purple, while other granules show a faintly pink color. In the pollen mother cell of *Lilium* all cytoplasmic granules are stained a fairly deep red-purple.

A remarkable fact is that the degree of coloration in the metaphase spindle varies in different materials. In the pollen mother cell of *Lilium*, the spindle fibers are stained even more intensely than the cytoplasmic granules (Fig. 9). On the other hand, in the root tip cell of *Allium* the metaphase spindle shows a faint pink color. Moreover, in the root tip cells of *Lilium*, *Zea*, *Pisum*, and *Vigna* the spindle is nearly colorless (Fig. 11).

In all the cells investigated, the interzonal region and the phragmoplast are almost entirely negative in their staining reaction for polysaccharides. The cell plate is strongly positive, however, and does not show any granular structure, appearing as a lamella accompanied by diffusedly stained thin layers on both sides (Figs. 10 and 12).

DISCUSSION

Concerning pentosenucleic acid in the achromatic organelles (Table I), some investigators using animal material reported the presence of pentosenucleic acid in the metaphase spindle, whereas others obtained opposite results. For instance, Stich [27] detected pentosenucleic acid in the poleward region of the spindle, whereas this was not the case with Jacobson and Webb [8] and Davies [4]. The cause of this discrepancy might relate to the difference of materials.

As far as the present authors could observe, pentosenucleic acid was contained in the spindle and in the phragmoplast in all the plant cells investigated.

The shapes of the polar caps, the spindle and the phragmoplast in preparations fixed in Carnoy's or Telyesniczky's fixative are similar to those *in vivo*, although a slight deformation is caused by the fixatives. This fact suggests that the structures of these mitotic organelles are fairly rigid.

The polar caps of a living stamen-hair cell of *Tradescantia* appear to be merely protuberances of the nuclear cavity free from granules which are transformed into the apices of the spindle [31]. After fixation and staining, the polar caps show a fibrous structure and give the staining reaction for pentosenucleic acid in contrast to the part of the nuclear cavity which contains no stained material except the chromosomes.

The poleward regions of the spindle in metaphase and anaphase also appear hyaline and contain no granule *in vivo*; in fixed and stained prepa-

rations they show a fibrous structure and give the staining reaction for pentosenucleic acid.

The phragmoplast *in vivo* (in the stamen-hair cell of *Tradescantia* and the pollen mother cell of *Lilium*) is a hyaline body free from granules which at first assumes a spherical or a barrel-like shape, gradually becoming flattened and taking the form of a convex lens. After the cell plate has reached the side wall, the phragmoplast takes the shape of a concave lens and small granules appear in the central region; the hyaline areas therefore are present only near the side wall and eventually fade away. These changes in the shape of the phragmoplast *in vivo* are almost the same as those of the fixed phragmoplast. In the present study the fixed phragmoplast appears as a fibrous body and has an intense staining reaction for pentosenucleic acid.

In a living pollen mother cell of *Lilium* in prometaphase the chromosome group is enclosed in a granule-free hyaline area ([23], Fig. 22). In fixed material this area shows irregularly distributed fibers and here pentosenucleic acid is indicated by an intense staining reaction.

From the above-mentioned facts it can be concluded that the polar caps, the hyaline area around the chromosome group in prometaphase, the mitotic spindle in metaphase, the poleward regions of the spindle in anaphase, and the phragmoplast have in common at least the following three characteristics, viz., (1) a hyaline appearance in living state; (2) a fibrous texture in fixed preparations; and (3) an intense staining reaction for pentosenucleic acid.

The present investigation proved that the regions where the formation of the spindle, the movement of the chromosomes and the formation of the cell plate take place are the regions containing pentosenucleic acid in large amounts. The localization of pentosenucleic acid suggests that this substance is connected with the activities of the mitotic organelles. In relation to this point, Stich's opinion [30] is worthy of note; he considered that the mitotic spindle consists essentially of a pentosenucleoprotein complex of long molecules and he offered an hypothesis about the relation of pentosenucleic acid to the development and structure of the spindle.

In anaphase, the interzonal region of the mitotic spindle is considered to differ largely in composition from that of the poleward regions.

The senior author [23] found by centrifuge experiments that the structure of the mitotic spindle had been partially dedifferentiated in anaphase, i.e., the interzonal region is less resistant against reformation of the spindle than the poleward regions.

Nakamura [17, 18, 19] observed the stamen-hair cell of *Tradescantia* and the pollen mother cells of *Lilium*, *Fritillaria*, etc. with polarized light *in vivo* and in the fixed state. According to him, the equatorial region of the spindle in anaphase, i.e., the interzonal region, does not show any recognizable birefringence, while the poleward regions exhibit a birefringence of positive sign in respect to the spindle axis. Gradually a positive birefringence appears in the interzonal region when it is occupied by the phragmoplast. He suggested the possibility of a change or replacement of the substances in the interzonal region at the time of the formation of the phragmoplast. Inoué [6, 7] also studied the pollen mother cell of *Lilium in vivo* with polarized light and found that the interzonal region is nearly isotropic, while the poleward regions show a strong positive birefringence. But he observed some weakly birefringent fibers (or fibrils) in this isotropic "gap" and considered these fibers to be those connecting the two spindle poles. In telophase the connecting fibers increase in number and in birefringence in the equatorial region and finally form the phragmoplast.

Little is known of the interzonal region wherein the phragmoplast emerges. It remains one of the most obscure things about the mitosis of plant cells.

In the observation *in vivo* with both ordinary and phase-contrast microscopes, the interzonal region is difficult to see in detail, being confused by numerous granules which come into sight immediately after the separation of the daughter chromosome groups.

In the present investigation, the interzonal region shows various types of artefact and at the same time gives nearly the same degree of staining reaction for pentosenucleic acid as the cytoplasm.

Jacobson and Webb [8] found an intense accumulation of ribonucleoproteins (pentosenucleoproteins) in the interzonal region of the fibroblast and the osteoblast, cultured *in vitro*, of chick and mouse. According to them, ribonucleoproteins in this region appear granular and are presumed to be shed from the separated daughter chromosomes into the interzonal region. Davies [4] observed the living chick fibroblast *in vitro* with ultraviolet light *in vivo* and noticed a higher concentration of the material absorbing ultraviolet rays in the interzonal region than in the cytoplasmic area.

In plant cells, in the present investigation, before the appearance of the phragmoplast there is no conspicuous accumulation of pentosenucleic acid in the interzonal region. A plentiful accumulation of pentosenucleic acid is seen for the first time in the phragmoplast, which is a characteristic mitotic organelle of plant cells, and the phragmoplast begins to develop after the daughter chromosomes contract at the poles.

Some investigators ([16], p. 106; [32], p. 154) have drawn attention to the fact that the phragmoplast is stained by basic dyes, while the cell plate region takes no stain.

The present authors have proved that pentosenucleic acid is a prominent chemical component of the phragmoplast and is responsible for its coloration by basic dyes, and that the cell plate region is free from pentosenucleic acid, but rich in polysaccharides. Pentosenucleic acid in the phragmoplast may be considered to play a role in the process of the cell plate formation, but pentosenucleic acid fades away in the region where the cell plate formation has been accomplished.

On the proteins of the spindle, a few experiments with pepsin were reported during the earlier period of investigation (cf. [14], p. 418). As in the case of pentosenucleic acid, hitherto few studies have been made on the proteins of the mitotic spindle and of the phragmoplast (Table II).

In plant cells, the mitotic spindle and the phragmoplast have the staining reaction for proteins in the same degree as cytoplasm when stained with any one of the bromophenol blue–mercury, ninhydrine-Schiff, and alloxan-Schiff methods.

Kaufmann *et al.* [9, 10] found that the stainability of the cellular structures with acidic dyes is raised after hydrolysis of pentosenucleic acid, and reversely, that the stainability with basic dyes is increased after hydrolysis of proteins. They explained these facts as follows, viz., that the molecules of pentosenucleic acid are combined with the basic groups of the protein molecules in the cellular structures, and removal of either of these components by hydrolysis provides new sites on the molecules for another component to form an attachment for the corresponding dye. Kaufmann *et al.* described the nature of chromosomes, nucleoli and cytoplasm, making no reference to the achromatic figure. They reported only the presence of pentosenucleic acid in the spindle.

The present authors applied Kaufmann's test to the mitotic spindle and the phragmoplast of plant cells and found that pentosenucleic acid in these organelles is associated with proteins.

Concerning polysaccharides in the mitotic organelles, reports so far published are also few. By staining methods, Monné and Slautterback [15], using the sea urchin egg, and Stich [28], using a variety of cells, found that the spindle, the centrosomes and the astrospheres contain polysaccharides. Stich [30] postulated that polysaccharides in the spindle and in the asters serve to make the fibrous texture of these mitotic organelles rigid and stable.

The present authors employed Lillie's periodate-Schiff method for stain-

ing polysaccharides and demonstrated a positive staining reaction in the metaphase spindle of the pollen mother cell of *Lilium* and in the cell plates of all plant cells investigated.

It is an interesting fact which deserves further study that the metaphase spindle of the pollen mother cell of *Lilium* contains polysaccharides, whereas the spindles of the root tip cells of *Lilium* and other plants do not. Stich [28] also observed in animal cells that the spindle gives the staining reaction for polysaccharides in various degrees.

The cell plate gives a strong staining reaction for polysaccharides, while the phragmoplast is negative; therefore it is conceivable that highly polymerized polysaccharides are produced for the first time at the site of cell plate formation. Furthermore, it seems that the production of these polymerized polysaccharides occurs at the equatorial plane of the phragmoplast before it develops and reaches the peripheral cytoplasmic layer on the side wall. The production of polysaccharides advances concurrently with the proceeding development of the cell plate.

The results obtained by staining methods are qualitative and are expected to be confirmed by more accurate and quantitative investigations.

SUMMARY

1. In the mitotic spindle and the phragmoplast of fixed plant cells, cytochemical tests with staining methods were carried out for pentosenucleic acid, proteins and polysaccharides.

2. In late prophase, pentosenucleic acid is accumulated in the polar caps. In the pollen mother cell of *Lilium* in prometaphase of the meiotic first division, pentosenucleic acid is present in a fibrous area around the chromosome group.

3. The metaphase spindle contains abundant pentosenucleic acid and is clearly distinguishable from the cytoplasmic area.

4. In anaphase the poleward regions of the spindle are proved to contain pentosenucleic acid as in metaphase; on the other hand, the interzonal region between the separated daughter chromosome groups gives the staining reaction for pentosenucleic acid in almost the same degree as the cytoplasm. The difference between the poleward regions and the interzonal region is discussed.

5. The phragmoplast is rich in pentosenucleic acid, but the cell plate region is free from it.

6. By staining with acidic dyes combined with hydrolysis of pentose-

nucleic acid, it was shown that pentosenucleic acid is associated with proteins in the polar caps, in the mitotic spindle and in the phragmoplast.

7. The cell plate clearly shows the presence of polysaccharides. Polysaccharides already appear at the equatorial plane of the phragmoplast before it reaches the side wall.

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THE INTRACELLULAR DISTRIBUTION OF METAL IONS IN RAT LIVER[1]

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Considerable interest has recently been centered upon the metal ion accumulating properties of subcellular particles, particularly mitochondria [1, 7, 11]. In the course of these and other studies [2, 12, 13], determinations of the metal ion contents of one or more cell fractions have been made, but they have not been done with any attempt at relating such data to other fractions or to the cell as a whole. The present study was undertaken to provide a more comprehensive picture of the distribution of sodium, potassium, calcium and magnesium among the cell fractions of rat liver, as prepared by differential centrifugation.

METHODS

Liver Cell Fractionation

The livers of nembutalized rats were perfused *in situ* with 500 ml of cold (2°C) 0.25*M* sucrose solution. The perfusion was done in a retrograde fashion through the thoracic portion of the inferior vena cava, with the infra-hepatic vena cava clamped, and the portal vessels severed. Portions of the liver which obviously contained residual traces of blood were discarded, along with trimmed-off hilar tissue. The remainder was pressed through a stainless-steel sieve; the resulting pulp was suspended in about 30 ml of cold 0.25*M* sucrose solution and homogenized for 5 minutes in a Potter-Elvehjem type homogenizer. The homogenate was diluted to 50 ml with cold sucrose solution.

The fractionation procedure was essentially that described by Schneider [9, 10]. The entire procedure was carried out in a cold room (0 5°C) or in a refrigerated centrifuge. The homogenate was centrifuged for 15 minutes at 600 gravity in two 50 ml lusteroid tubes placed in an angle rotor of an International Clinical Centrifuge. The sediment was resuspended in one tube with 5 ml of sucrose solution and centrifuged at 600 × gravity for 5 minutes. This was repeated twice more, the washings

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each time being added to the supernatant fluid from the original centrifugation. The resulting sediment was designated the *nuclear fraction*, even though it was contaminated with unbroken whole cells.

The supernatant fluid from the nuclear fraction was centrifuged for 7 8 minutes at 17,300 × gravity in the preparative rotor *A* of the Spinco model E ultracentrifuge. Two sedimented layers could be distinguished—the brown, well-packed mitochondrial pellet, and a poorly-packed pink layer overlying it. Although there is no general agreement as to the proper identity of the pink layer, it was decided here to include it in the microsomal fraction on the basis of studies carried out in this laboratory [4]. The pink material was pipetted off and recentrifuged, throwing down a small amount of mitochondrial material, which was added to the original batch. The combined mitochondrial pellets, designated the *mitochondrial fraction*, were washed twice, the second sedimentation being done at 9210 × gravity.

All the supernatant fluids from the above steps, including the pink material, were pooled and recentrifuged for 30 minutes with a force of 102,750 × gravity. The resulting pellets were resuspended in two tubes and recentrifuged for the same time at the same speed. The sediment was designated the *microsomal fraction*.

The supernatant fluid and washings from the microsomal fraction were combined and analyzed without further fractionation. This was designated the *supernatant fraction*.

Chemical Methods

Nitrogen.—Determinations of total nitrogen were performed by Kjeldahl digestion, followed by steam distillation of ammonia into a boric acid receiver, and titration of the resulting ammonium borate with standard hydrochloric acid solution to a methyl red end-point [8].

Ashing of tissue samples.—Aliquots of the cell fractions were wet-ashed with nitric acid and then perchloric acid. Most of the perchloric acid was evaporated off to reduce the acid content, but the ash was left moist in order to insure solubility, since it was found that complete drying resulted in a residue which was impossible to dissolve.

Potassium and sodium.—These elements were determined on aliquots of the ashed samples by means of a Perkin-Elmer flame photometer, using lithium as an internal standard.

Magnesium and calcium.—These elements were determined by titration after separation on an ion exchange column, as previously described [3].

RESULTS AND DISCUSSION

Nitrogen.—The results of nitrogen analyses are summarized in Table I. The percentage of the homogenate nitrogen found in each cell fraction is given. Data are given for young rats, which ranged in weight from 184 to 270 grams.

Metal ions.—The results of metal ion determinations on the various cell

TABLE I

Partition of nitrogen and metal ions among liver cell fractions.

Concentrations are expressed as micromoles per milligram of nitrogen. "%" refers to percentage of homogenate component found in cell fraction. All figures are given in terms of the mean ± standard deviation. Figures in parentheses are numbers of livers analyzed.

		Nitrogen	Potassium	Sodium	Calcium	Magnesium
Homogenate	Conc.	—	1.21 ± 0.24 (9)	0.400 ± 0.090 (7)	0.058 ± 0.011 (5)	0.331 ± 0.025 (8)
Nuclear fraction	Conc.	—	0.87 ± 0.09 (8)	0.308 ± 0.082 (6)	0.064 ± 0.011 (4)	0.334 ± 0.049 (5)
	%	15.0 ± 5.1 (7)	13.6 ± 7.4 (8)	12.5 ± 4.7 (6)	15.5 ± 2.9 (4)	13.4 ± 4.0 (5)
Mitochondrial fraction	Conc.	—	0.87 ± 0.14 (7)	0.124 ± 0.073 (4)	0.109 ± 0.011 (3)	0.294 ± 0.028 (4)
	%	26.2 ± 3.0 (5)	18.4 ± 6.1 (7)	6.8 ± 2.1 (4)	47.7 ± 1.3 (3)	21.8 ± 3.5 (4)
Microsomal fraction	Conc.	—	0.35 ± 0.14 (7)	0.147 ± 0.021 (4)	0.043 ± 0.008 (5)	0.442 ± 0.048 (6)
	%	36.9 ± 4.0 (7)	11.9 ± 3.0 (10)	14.4 ± 2.5 (7)	23.7 ± 3.8 (7)	48.0 ± 4.5 (9)
Supernatant fraction	Conc.	—	3.67 ± 0.57 (7)	1.45 ± 0.33 (4)	0.130 ± 0.052 (5)	0.244 ± 0.030 (6)
	%	17.7 ± 1.9 (7)	52.7 ± 8.9 (10)	65.6 ± 7.9 (7)	26.6 ± 8.0 (7)	12.8 ± 3.4 (8)

fractions are given in Table I. There are given for each cell fraction, first, the metal ion concentrations in micromoles per milligram of nitrogen of that fraction; and second, the percentage of the metal ion of the whole homogenate found in that fraction.

It is seen first of all that the four metal ions fall into two general categories: (*a*) potassium and sodium, which appear to the extent of about 50 per cent in the supernatant fraction, and (*b*) calcium and magnesium, which are found chiefly in the particulate fractions. This result is broadly consistent with the known behavior of these metal ions with respect to their reactions with protein molecules [5]. That is, calcium and magnesium ions are commonly "bound" or complexed in a non-diffusible form by proteins, while potassium and sodium ions are expected to form such compounds weakly, if at all. Even if the latter two ions were associated with the particulate fractions in the living cell, one would expect them to diffuse at least to some extent out into the supernatant fraction during the fractionation. Calcium and magnesium, on the other hand, remain bound to the proteins of the insoluble fractions.

It may be noted that the quantity of potassium found in the supernatant fraction is a minimum figure, since some of the intracellular potassium is probably washed out during the perfusion of the liver. On the other hand, the sodium and calcium values for this fraction are probably maximum ones, since there is undoubtedly contamination with sodium and calcium of extracellular origin which escaped being removed by perfusion. A more detailed examination of this point will be made in a later section.

Several other points concerning the distribution of ions among the particulate fractions may be mentioned. Considering first the *concentrations* of metal ions, it is seen that potassium is found in rather low concentration in the microsomal fraction, as compared to that in the nuclei and mitochondria. Sodium, on the other hand, has its lowest concentration in the mitochondrial fraction.

Turning to *contents* (expressed as percentage of homogenate ion found in the cell fraction), the mitochondria are seen to contain more potassium and calcium than the other particulate fractions, while the microsomes contain larger shares of sodium and magnesium. The microsomes, in fact, contain nearly half the magnesium found in the whole homogenate.

The question arises whether the distribution of any of the ions among the cell fractions may be accounted for on the basis of a uniform distribution in the water of the fractions. If any ion is freely diffusible under the conditions of the fractionation, it should be evenly distributed in the total water

of the pellet (including the water associated with the particles themselves and the interstitial water) and in the water of the supernatant fluid in each step. On the basis of the relative volumes of the pellet and supernatant fluid in each step, it can be calculated that none of the four ions measured fulfills these conditions.

To test further the non-diffusibility of the metal ions associated with the particles, the following experiment was done. Two rats were prepared in the usual manner and the homogenate mixed thoroughly. The homogenate was then divided into two equal portions designated *A* and *B*, and the two portions fractionated simultaneously in the usual manner. In addition, each fraction from portion *B* was subjected to two further washings and recentrifugations in fresh sucrose solution. On each of the particulate fractions, the metal ion and nitrogen contents were determined.

It was found that two additional washings have only a minor effect upon the concentrations of any of these constituents. Thus the metal ion content of the particulate fractions is greater in all cases than that which would be expected if the ions were uniformly distributed solutes.

Apparently, then, the ions are present in the particles in a non-diffusible form, hindered in some manner from leaving the particles, although what mechanism is operative here only further experimentation can tell. It may be surmised that although those metal ions found associated with the particles are not necessarily the full complement of ions in the particles of the living cell, yet they may be considered as a special and characteristic group of these, and as such may have significance in the comparison of normal with various abnormal physiological states.

An approximation may be made to the magnitude of these metal ion complements of the particulate fractions compared to the total intracellular metal ion content of the intact cell. Calculations based on the figures given by Lowry *et al.* [6] (derived by the deductive histochemical method) reveal that roughly 100 per cent of the total calcium of the cell is recovered in the particulate fractions. Thus, the calcium found in the supernatant fraction is probably of extracellular origin. Of the magnesium, about 60 per cent is found in the particles and an additional 10 per cent in the supernatant fraction; and of potassium, about 10 per cent remains in the particles, with 10 per cent being found in the supernatant fraction. The ions making up the difference between these values and 100 per cent were apparently washed away during the perfusion, as was reasoned earlier. Comparison of sodium cannot be made, since Lowry *et al.* assumed that no sodium was present in the cells.

SUMMARY

Rat liver cell fractions were prepared by differential centrifugation, and analyzed for their content of nitrogen, potassium, sodium, calcium, and magnesium. Characteristic distribution patterns of the metal ions have been established for the cell fractions prepared under the conditions described.

Significant proportions of the metal ions of the cell are found to be associated in a non-diffusible form with the sedimentable cell fractions. It is suggested that these metal ions may be a special and characteristic group of the total of such ions present in the cell, although their specific functions are as yet unknown.

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MORPHOLOGICAL AND FUNCTIONAL STUDIES OF THYROID TISSUE CULTURES[1]

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The technique of tissue culture applied to the thyroid gland [4] has provided a method of studying the growth and behavior of this tissue. The first studies included the isolation of a pure strain of chick embryo thyroid cells [6] and observations that the anatomy and, to some extent, the function of cultivated thyroid tissue resemble those of the gland *in vivo*. This technique has also been applied to the study of the thyroid stimulating hormone (TSH) both as regards its morphological and physiological effects on the thyroid [9] and conversely its fate in the presence of thyroid tissue [13]. Considering the successful application of radioiodine in the study of thyroid biochemistry *in vivo*, in insolated whole glands or slices, it was felt that a reappraisal of thyroid tissue cultures using I^{131} would be of interest. The present study deals with (1) the morphological characteristics of thyroid fragments and their outgrowths in cultures, (2) the functional capacity of such fragments as determined by the concentration of I^{131} in the tissue and the ability to convert inorganic iodine to the organic form, and (3) the effect of TSH on the anatomy and function of the cultivated thyroid tissue.

MATERIALS AND METHODS

Morphological studies.—The thyroid glands of young adult rabbits were removed under nembutal anesthesia, bathed in tissue culture medium (to be described below) and cut into small rectangular fragments measuring about 1 mm in greatest diameter, and weighing about 0.5 mg. Three fragments were then attached to the sur-

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face of a No. 1 coverslip by means of a drop of chicken plasma which was allowed to clot. Two coverslips were then affixed to the flattened surfaces of the lower portion of a 150 mm pyrex tube, also by means of a plasma clot.

One ml of tissue culture medium was thereupon added. This consisted of a mixture of the following constituents: 40 per cent Gey's solution, a balanced salt solution [12], 20 per cent chicken embryo extract obtained from 10 day old embryos, 30 per cent horse serum, and 10 per cent human placental serum. To the Gey's solution was added an amount of penicillin and streptomycin to yield a final concentration in the complete medium of 40 units and 40 mg per cc respectively. The medium was completely replaced every fourth day with freshly prepared material. The culture tubes were then rotated in a roller drum at 7 r.p.h. in an incubator kept at 37.0°C.

At periodic intervals the tissue outgrowth was observed directly through a binocular microscope. At the end of the experiment the tissue was fixed in Bouin's solution and stained with hematoxylin and eosin, or fixed with methyl alcohol, followed by a methylene blue stain.

Radioautography.—One microcurie of carrier-free I^{131} was delivered to the tissue culture medium after six days of incubation. After 24 hours exposure to the radioiodine, radioautographs of the tissue fragments and their outgrowths were prepared. Both whole mounts and serial unstained sections were employed. Stripping film techniques as outlined by Fitzgerald [8] were utilized. Paraffin sections varied in thickness between 4 and 10 μ, while the length of exposure to the stripping film ranged from 12 to 72 hours.

I^{131} content of fragments.—By means of a plasma clot, 20 fragments of thyroid tissue were attached to a loose coverslip which was placed in the bottom of a culture tube. Two cc of bathing media were added. Three varieties of media were employed: (1) the complex tissue culture medium, as described above and employed in the morphological studies, (2) Gey's solution alone with added penicillin (40 units/cc) and streptomycin (40 μg/cc), (3) autologous rabbit serum with antibiotics added in similar concentration. At the beginning of the experiment 0.1 μc carrier-free I^{131} in 0.1 cc normal saline was added to the medium, which was not changed in these experiments.

At designated intervals the slides bearing the tissue were removed from the bathing medium, placed in sterile tubes, and measured for radioactivity in the well of a scintillation counter. Because a certain amount of medium was thought to adhere to the surface of the tissue, the radiation counts emanating from 20 fragments of muscle, of similar size and handled identically, were subtracted from the thyroid tissue values. The difference was thought to represent the minimal uptake of I^{131} by the thyroid fragments.

Chromatographic analysis of tissue hydrolysates and bathing medium.—Chromatographic analysis [14] of the thyroid fragments was performed after a 48-hour hydrolysis with crude tryptic enzyme carried out at pH 8.6 and 37°C. Ascending *n*-butanol-acetic acid (78 per cent *n*-butanol, 17 per cent H_2O, five per cent glacial acetic acid) and *n*-butanol-dioxane (80 per cent *n*-butanol, 20 per cent dioxane)–2*N* NH_4OH systems were employed for paper chromatography, using Whatman No. 1 paper. Carriers of iodide, monoiodotyrosine, diiodotyrosine, thyroxine and triiodothyronine were added in amounts sufficient for color reactions. Bathing medium was analyzed in similar fashion but without preliminary hydrolysis. The dried paper strips were

fed through an automatically recording rate meter, and the position of the radioactive components compared with the position of the carriers. The relative distribution of the radioactivity among the various organic components was determined by planimetry.

RESULTS

Morphological studies.—Two major cell types arose from these cultures, fibroblasts and epithelial cells. About one half of all cultures showed epithelial growth, and of these the large majority also showed proliferation of fibroblasts. In one sixth of the cultures, only fibroblasts were seen, while the remaining third failed to yield any cellular outgrowth at all. Good growth was observed to occur throughout the period of observation. Once an epithelial membrane had established itself, fibroblasts were never seen to outgrow it. There was no evidence of follicle formation or the deposition of extracellular colloid in the outgrowth.

Examination of serial sections of the central fragments revealed that cultivation was almost always accompanied by the appearance of patchy areas of necrosis, fibrosis, and hyalinization, with good preservation of follicular structure in other areas. The degree and extent of morphological preservation of structure did not correlate directly with the length of incubation.

No difference was noted in the morphology of thyroid explants cultivated for five days in the presence of 3 units of TSH (Thytropar, Armour, Lot No. M 1004) as contrasted with fragments grown in a control medium.

Radioautography.—After incubation in the presence of I^{131}, the central fragment with its surrounding outgrowth caused an intense blackening of stripping film exposed for 24 hours. In order to evaluate separately the contribution of the central fragment and the cellular outgrowth the following experiment was performed. Five days after explantation the central fragment of two cultures were dissected free of their outgrowths and replanted on separate coverslips in tubes containing 1 μc I^{131}. The outgrowths devoid of their mother fragment continued to be incubated in a medium also containing 1 μc I^{131}. In both instances the incubation was carried on for an additional 24 hours. After 14 days exposure to stripping film the outgrowths did not cause significant reduction of the emulsion. Sections of the central fragments, however, yielded good autographs with as little as 12 hours' exposure to the film.

Fig. 1 illustrates a radioautograph of section of the central fragment of rabbit thyroid incubated for six days, the last day of which, in the presence

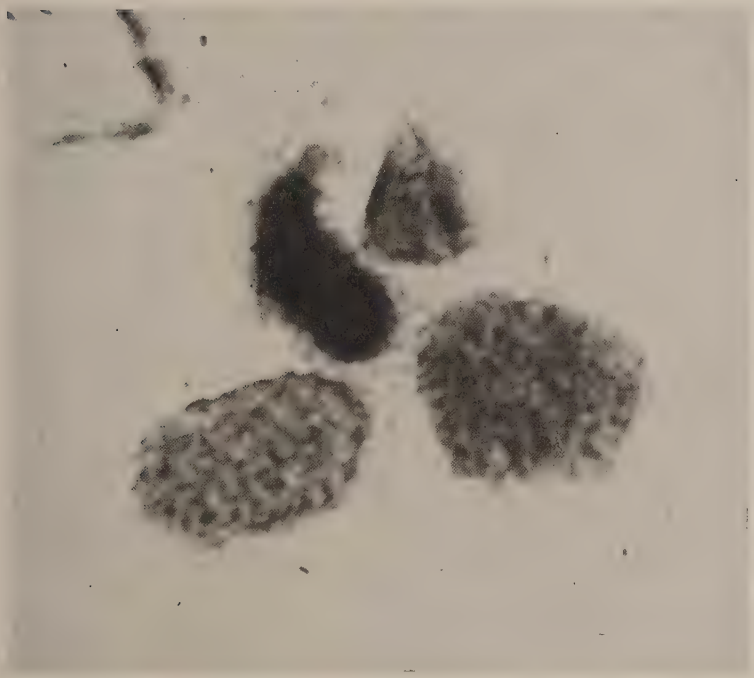

Fig. 1. Radioautograph of section of thyroid fragment cultivated for six days, exposed to 1 μc I^{131} for one day.

of I^{131}, and exposed to stripping film for three days. The radioiodine is seen to be concentrated in the follicular spaces.

In all instances controls, treated similarly except for the addition of isotope, were not observed to cause spurious chemical reduction of the silver bromide crystals in the emulsion.

I^{131} content of thyroid fragments.—Fig. 2 illustrates the general nature of the iodine uptake curve in one representative experiment. Maximal uptake did not occur until 40–70 hours, after which the I^{131} content of individual cultures either remained at plateau levels or declined gradually. Since no attempt to standardize the thyroid fragments by weight was made, a large variation in the extent of maximal uptake, from 10 to 40 per cent of administered I^{131} was encountered. There appeared to be no significant difference in the shape of the I^{131} uptake curves regardless of which medium was

used, the complex organic medium employed in the morphological studies, Gey's solution, or autologous serum.

In order to assay the effect of the thyrotropic hormone on this system, three to 10 units of Thyrotropar (Armour) were added to the culture tubes 65 hours after the start of incubation. At this time the fragments were presumed to have accumulated maximal or near-maximal amounts of I^{131}. Determinations of I^{131} content were made at 1, 2, 24, and 49 hours, after the addition of TSH. The mean difference in I^{131} uptake after the addition of TSH and the 64-hour value was then computed. In Table I are indicated the combined results of three experiments in which autologous serum was employed as the bathing medium. As can be seen, there was no significant difference (at the $p = 0.1$ level) between cultures to which TSH was added and those serving as controls. Similarly, no difference was detected when Gey's solution served as the bathing medium.

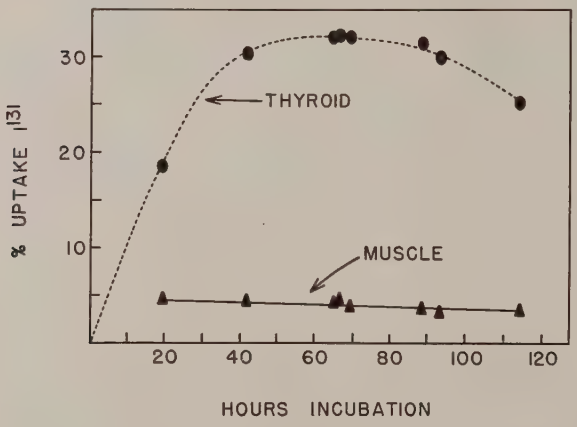

Fig. 2. Per cent uptake I^{131} of thyroid and muscle fragments.

Chromatographic analysis of tissue hydrolysates and bathing medium.— Table II summarizes the pertinent data on tissue hydrolysates. It will be noted that in three instances a radioactive peak falling into the region of monoiodotyrosine and diiodotyrosine did not allow resolution into the component amino acids. Furthermore, the amount of monoiodotyrosine always exceeded the amount of diiodotyrosine when these substances were distinguishable. No consistent effect was noted on the addition of TSH.

Analysis of the bathing medium uniformly failed to show the presence of any free iodinated amino acids. Frequently, however, origin material was

TABLE I

Variation in I^{131} uptake values.

TSH added at 65 hours			Control samples		Evaluation of difference between TSH and controls	
Hours after TSH added	*n*	Mean variation from 64-hour value*	*n*	Mean variation from 64-hour value*	*s*	*p*
1	9	−0.48	10	−0.16	0.55	0.2>0.3
4	10	−0.19	10	−0.34	0.93	0.2>0.3
24	10	−2.35	10	−2.66	2.77	0.8>0.9
27	4	−4.12	4	−2.15	1.81	0.1>0.2
49	3	−6.23	4	−6.72	3.38	0.8>0.9

n = number of cultures employed.
s = standard deviation of all determinations.
p = probability of accounting for the difference in variation by chance alone.
* As expressed in difference in per cent uptake.

TABLE II

Chromatographic analysis of tissue hydrolysates.

Medium	TSH units	Hours incubation			% I^{131} uptake	% organic iodine present as			
		with I^{131}	with TSH	Total		O	MIT	DIT	T_4
TC	–	48	–	192	15.1	8.2	46.7	23.6	20.7
TC	5	48	24	192	26.7	9.5	56.3	27.6	6.7
TC	5	96	24	96	12.7	6.9	36.5	35.8	18.3
TC	–	96	–	96	9.5	8.4	– 68.9	–	19.4
Gey's	5	96	24	96	18.9	5.7	– 73.0	–	20.1
Gey's	–	96	–	96	28.2	4.8	– 80.4	–	5.9

TC = Tissue culture medium, same as for morphological studies.
MIT = Monoiodotyrosine.
DIT = Diiodotyrosine.
T_4 = Thyroxine (including triiodothyronine, if present).

demonstrated on the chromatograms, which in one instance was shown to move with the mobility of α_1–α_2-globulins on electrophoresis. This substance was thought to represent thyroglobulin.

The addition of small (0.003 and 0.03 u. Thytropar, Armour) and relatively large (5 u., Lot No. 098802, Parke, Davis) quantities of TSH failed to effect the release of thyroxine or any other free iodinated amino acid into the medium, which was examined up to 24 hours after the addition of TSH.

The possibility that the plasma clot surrounding the fragments might adsorb TSH or liberated thyroxine was tested by the following experiment. Twenty loose fragments were dropped into the medium (tissue culture fluid, Gey's solution, with and without TSH), but the bathing fluid again failed to show the presence of thyroxine.

A single experiment was performed to test the possibility that the tissue culture medium might possess inhibitory factors to TSH. The following substances were assayed for thyrotropic activity by the chick thyroid weight technique: (1) tissue culture medium, (2) medium containing 1 mg (5 u.) TSH (Parke, Davis) freshly added, and (3) medium exposed to 20 fragments of thyroid for eight days and TSH for one day. There was no demonstrable inhibition of thyrotropic activity, either by the medium or the thyroid fragments.

DISCUSSION

It is of interest to examine the degree of differentiation of the thyroid epithelial outgrowth.

In these experiments it was not found possible to demonstrate follicular formation or colloid deposition in the epithelial sheets. Ebeling [6] reported the appearance of follicles and colloid in his chick embryo cultures, but this observation was challenged by Demuth [5].

The fact that the epithelial outgrowth did not yield an autograph after 14 days' exposure to the stripping film is not in itself surprising. In the central fragments radioiodine was seen to accumulate only in the follicular spaces, and as pointed out above, follicles were not formed by the epithelial outgrowth. It would be of interest to determine by combined isotopic and chromatographic techniques whether this outgrowth possessed any functional differentiation whatsoever.

As regards the functional aspects of these studies, it has been shown that the thyroid tissue can be kept in a biochemically active state for at least six days *in vitro*. Certain differences between the behavior of these preparations and the intact gland stand out: (1) a generally slower rate of iodine uptake than would be expected in the intact gland, (2) a higher ratio of monoiodotyrosine to diiodotyrosine than is encountered *in vivo* after similar

exposure to I^{131}, (3) the histological evidence of degenerative changes *in vitro*, (4) the failure to demonstrate the liberation of the thyroid hormone, and (5) the failure to respond to TSH.

In regard to the last point, various parameters were used to test the effect of TSH on the system employed.

1. The I^{131} content of the thyroid fragments seemed not to be influenced by the addition of TSH, as judged within the limits of the experimental techniques employed.

2. The failure to demonstrate release of thyroxine into the medium when TSH was used would preclude a change in the radioiodine content of the tissue, if one accepted the hypothesis that the primary effect of TSH involves the release of the hormone. The decline in the iodine content of the tissue fragment after 70 hours' incubation, frequently seen in both TSH and control tubes as illustrated in Fig. 6, can probably be attributed to a certain amount of tissue disintegration with release of thyroglobulin. This substance, in all likelihood, represents the origin material seen in chromatograms of the bathing medium.

Like the results reported here, Taurog, Potter, and Chaikoff [15] have indicated their inability to demonstrate free thyroxine in the bathing medium of thyroid slices. Furthermore, TSH did not influence the metabolism of their mitochondrial system.

3. The failure to demonstrate morphological changes in the central fragments after exposure to TSH corroborates the other negative observations. Demuth [5] also could not demonstrate any certain morphological change in his tissue culture preparations on the addition of TSH.

In addition to these experiments preliminary work has also tended to show that TSH does not appear to alter the disappearance of glucose from the medium which occurs during the incubation of thyroid fragments.

It is impossible, however, to ignore the substantial body of literature which supports the belief that TSH–thyroid interaction does in fact occur *in vitro*. Eitel, Krebs and Loeser [7] reported characteristic morphological changes in thyroid slices when incubated in the presence of TSH. An increased oxygen consumption by thyroid slices following the addition. of TSH *in vitro* has also been reported [1, 3, 11]. Rawson *et al.* [13] demonstrated the inactivation of TSH by thyroid tissue *in vitro*. It should be noted that in this work a much larger ratio of thyroid tissue to TSH was employed than in the experiment cited in this paper. Junqueira [9] reported an increased number of intracellular colloid droplets in thyroid fragments after the application of TSH. Morton and Schwartz [10] indicated that TSH in-

creased the rate of incorporation of labeled phosphorus into the phospholipid fraction of thyroid slices.

Recently Botkin and associates [2] have published the results of their investigation on the *in vitro* action of TSH. According to these authors, the I^{131} uptake of whole rat glands after four hours' incubation in a modified Krebs buffer solution containing 10 units of TSH was only one third that of specimens incubated without TSH. Preliminary studies in our laboratory have tended to confirm these results.

Whether the failure to respond to TSH in the experiments detailed in this paper is due to the state of small subdivision of the glands, to some peculiarity of the media employed, or to other variants in the experimental conditions has not been determined. The possibility exists that the inability to respond to TSH merely reflects a diminished functional capacity of the fragments compared to the activity of glandular tissue *in situ*.

SUMMARY

1. By the use of the roller tube technique of tissue culture it was found possible to demonstrate the outgrowth of epithelial membranes from 50 per cent of tissue fragments of adult rabbit thyroid.

2. Radioautographs of thyroid fragments prepared by the addition of radioiodine *in vitro* showed localization of I^{131} in the follicles. There was no apparent concentration of iodine in the epithelial outgrowth.

3. Maximal I^{131} uptake by 20 rabbit thyroid fragments, weighing about 10 mg altogether, ranged between 10 and 40 per cent and was achieved between 40 and 70 hours of incubation.

4. Enzymic hydrolysis of tissue fragments with subsequent paper chromatographic analysis yielded evidence of the presence of monoiodotyrosine, diiodotyrosine, and thyroxine as constituents of the iodo-protein formed.

5. TSH was not found to influence the I^{131} content of the thyroid fragments, nor was it possible to demonstrate the release of free labeled amino acids into the medium. No histological changes were observed after the exposure to TSH.

6. The advantages and limitations of the tissue culture technique for the purpose of studying *in vitro* iodine metabolism of thyroid tissue are discussed.

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INFLUENCE OF WATER CONTENT ON UV-INDUCED S-MUTATION AND KILLING IN *SERRATIA*

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The study of the influence of secondary factors on the radiation-induced mutation process may be a way to learn about the nature of the part of the cell where the microphysical event(s) occur which lead after further amplification reactions to the mutation. Many years ago Stadler [26] observed that germinating seeds give a higher X-ray mutation rate than dry ones. Working with pollen and seeds of *Antirrhinum*, Kaplan [5, 6] and Knapp and Kaplan [19] demonstrated that this increase of the X-ray induced mutation rate in soaked cells is at least partially reversible by redrying and therefore mainly due to the higher water content. They also found a smaller increase in chromosomal mutations (not in factor (point) mutations) under anaerobic than under aerobic conditions. Later results on the influence of oxygen on the effects of ionizing radiation (see [21]) strongly suggested the hypothesis that part of the radiation energy is absorbed first by water and produces from it radicals like OH, HO_2, or H_2O_2, which react with sensitive cell structures. According to this theory a higher water content would give a greater chance to form such radicals and hence would increase the induced mutation rate.

From the study of the action spectrum of uv-induced mutations (see [21]; for *Serratia* [10]) it is known that germicidal uv is essentially not absorbed by water, but that probably nucleic acid is the target structure for the initiation of the mutation process. Therefore, a different response of uv-induced mutations to changes in the water content is to be expected than is found with X-ray induced mutations. The same should be true for the bactericidal effect of uv which shows a similar action spectrum. A stronger killing by uv of dry bacteria (e.g. *E. coli*) than of wet ones was first reported by Fair and Wells [1]. This was not confirmed by Rentschler and Nagy [22], perhaps because of the inaccuracy of their method.

On the following pages experiments are reported in which cells of varied water content were exposed to uv. The bacterium used, *Serratia marcescens*,

is especially suitable since a high percentage of color mutations is induced by uv so that no screening, such as is necessary with rare mutations, is required; mutant and parent colonies can be counted on the same plate. These mutations appear not as colonies changed *in toto*, but as red colonies with sectors or spots of white or pink mutant type. As reported earlier [8, 10, 11, 12], this sectoring is a special type of mutation delay and probably due to a very mutable state of the genetic material. Though the cause of the appearance of the new mutants as sectors is not yet completely clear (as is true in all known cases of "delay of mutation") the sector (*s*) colonies arise from cells with a mutated genotype and can be used for studies of mutation induction.

Strain and mutant types

Strain K of *Serratia marcescens* (*Bacterium prodigiosum*) used by the author since 1945 is unusually mutable. The main characters (see [7–12, 14–17]) may be summarized as follows: The carmine red (*r*) normal type gives rise frequently to nearly white (*w*) colonies, more rarely to pink (*h*) and dark red (*d*), and seldom to yellow (*g*), violet (*v*), cinnabar (*c*), and orange (*o*) types. A subculture of a 48-hour old *r*-colony spontaneously contains, besides *r*, about 2 to 5 per cent *w*, less than 0.1 per cent of the other types, and an average of 2.11 ± 0.11 per cent *s*-colonies. These *s-colonies* consist of several spots or sectors of *w* (rarely also some *h* or other types) in addition to the *r*-type.[1] Subcultures of them contain, besides *r*, 10 to 50 per cent *w* (in some cases also a few percent *h*) and an average of 4.23 ± 0.19 per cent *s*-colonies. Subcultures of *w*-colonies contain, besides *w*, 20 to 25 per cent *r*, and an average of 2.07 ± 0.29 per cent *s*-colonies. When suspensions of *r*-cells are irradiated with uv the *s*-content among the survivors increases linearly with doses up to 15 or 20 per cent; then it falls. The *w*-content for the most part remains constant, sometimes decreasing or increasing slightly. The high content of the *w* (or *h*, etc.) mutant in *s*-colonies indicates that the mother cells of the *s*-colonies contain a changed genetic material compared with *r* or *w*, and hence are mutated. It seems that most *w*-mutants arose in previous generations during growth of *s*-clones in the parent type. The *s*-colonies are, for the most part, due to "fresh" mutations which occurred in *s*-cells shortly before plating.

The possibility that *s*-colonies are merely grown from clumps of *r* and *w*

[1] The "speckled" colony type of the HY strain of *Serratia* investigated by Labrum and Bunting [20] is not induced by uv and also has a different appearance than the *s*-colonies of the K strain used here.

cells has been ruled out [15, 18]. Two main mechanisms to explain the formation of *w*- and *r*-types during the growth of an *s*-colony have been considered and investigated [8, 9, 10, 11, 12]: (1) *segregation* from a partially mutated genetic material (heterocaryosis, diploidy, subdivided chromatids, free extra-chromosomal particles); (2) a *labile state* turning over to the *r* and the mutated *w* state during colony growth. The higher content of *s*-colonies in *s*-subcultures than in *r* or *w* progenies (significance by *t*-test: $t = 9.4$, $P = 10^{-13}$ for *s-r*; $t = 6.2$, $P = 10^{-9}$ for *s-w*) is an indication that the partially mutated or labile *s*-state is a condition of the autosynthetic (=genetic) material. Several observations favor the labilization hypothesis (2) but cannot rule out irrefutably a complicated case of segregation (1). These facts are: (*a*) the resting cells contain mostly only one visible nucleoid; (*b*) the variance of the *w*-content of *s*-subcultures is much higher than normal ($\chi^2_{27} = 530$), therefore, no fixed proportion of mutated material is regularly segregated; (*c*) the frequency distribution of the number of mutant sectors in *s*-colonies does not fit simple cases of segregation from subdivided chromatids or free extra-chromosomal particles, but agrees with the labilization hypothesis; (*d*) when a suspension containing many *s*-cells induced by uv-irradiation is spread on a nutrient agar and then kept in the cold for various times until incubation, the percentage of *s* decreases while that of *w* increases during a period when the viable count remains constant.

For the present work the *mechanism* of sector formation is of only secondary importance since this "mutation delay" occurs *after* the induction of the mutation in the *s*-cells, and probably is a late step in the reaction chain from the hit to the perfect stable mutation. The main subject of this report is the *induction* of the *s*-mutation, as well as killing, as influenced by the water content during uv-irradiation.

METHODS

To obtain *cells of different water content* the bacteria were exposed to atmospheres of different relative humidity. For this purpose a series of glass plates (cut microscope slides), 15 × 25 mm, were covered with a film of gelatin (Difco, Bacto). On the dry film 0.01 ml of a dense suspension in distilled water of *r*-cells from three-day old surface colonies (ca. 10^9 cells per ml) was evenly spread with a micro-pipette, then the plate was placed in a desiccator over $MgCl_2 \cdot 6\ H_2O$ yielding 33 per cent relative humidity (r.h.) at room temperature (about 25°C). After drying, the plates were put into irradiation chambers. These were glass dishes 55 mm in diameter and 20 mm high containing 15 ml of a salt solution and an aluminum bench to hold 3 inoculated glass plates in the atmosphere ca. 4 mm over the solution. The salt solutions used were, for 33 per cent r.h., concentrated $MgCl_2 \cdot 6\ H_2O$; for 50 per cent r.h., 95 g $MgCl_2 \cdot$

6 H_2O + 100 ml H_2O dist.; for 75 per cent r.h., concentrated NaCl; for 97.5 per cent r.h., 4.5 per cent NaCl solution. (100 per cent r.h. was not used because of the condensation of water on the plates.) The chambers were made air tight with covers of 2 mm thick quartz plates, or, in the cases of unirradiated controls, glass plates. Gelatin plates spread with cells were allowed to remain for $1\frac{1}{2}$ to 2 hours in the chambers to ensure the adjustment of the water content of the cells to the r.h. (special experiments had shown that the water uptake was accomplished already after about $\frac{1}{4}$ hour). Then the chambers were placed under the 2 cm wide opening of a Westinghouse germicidal lamp, and the cells irradiated at a distance of 11 cm (about 4 ergs/mm²/sec). Various doses were obtained by successively flapping lids over the 3 plates after a certain exposure time. After irradiation all plates were put for 10 min into a moist chamber with 97.5 per cent r.h. to remoisten the cells slowly. All these operations were done at room temperature. Then each plate was dropped into a test tube with 5 ml saline at 37°C and shaken for 10 min to dissolve the gelatin and suspend the cells. Finally suitable dilutions of the suspensions were plated on synthetic agar for growth (for composition see [10]). After 2 days incubation at 30°C the normal and mutant colonies were counted. Since on crowded plates the frequency of *s*-colonies is usually found to be smaller, only plates with less then 500 colonies were scored.

One experimental series was done to test whether the speed of change in water content after uv irradiation would influence the results. To slow down the *reswelling* of dried (50 per cent r.h.) cells a plate was fixed in a small clamp at the end of a wire pierced through a cork stopper. The plate with this holder was put successively in a series of 6 test tubes containing $MgCl_2$ solutions of concentrations increasing stepwise up to 97.5 per cent r.h. The plate hung just over the salt solution and was kept for 10 min in each tube.

In the experiments on *photoreactivation* the chambers were put under 2 Westinghouse 15 Watt daylight fluorescent bulbs at a distance of 8 cm for 1 hour at room temperature. This lamp produced no appreciable warming of the suspension, and good reactivation was obtained.

RESULTS AND DISCUSSION

Water content and uv sensitivity.—Fig. 1 shows the results of one experimental series, Table I gives the actual values found in another series. The logarithm of the number of survivors follows about a straight line with dose. The *w*-mutants seem to increase sometimes, but not consistently in all experiments. The percentage of *s*-mutants increases nearly linearly, reaches a maximum around 10 per cent, and then seems to fall. From these curves can be concluded that killing as well as *s*-mutation are one-hit processes under these conditions, i.e. one uv quantum is able to start the processes leading to the observed effects. The slope of the linear part of the curves gives a measure of the mutation or killing rate. These rates increase with decreasing water content. Though a killing of 70 to 80 per cent of the cells due to the drying and remoistening procedure alone can be observed, e.g. Table IV,

selective killing cannot be responsible for the change of the rate with r.h.; for it does not vary with r.h. in the same direction as the rates (Fig. 1).

In Table II the ratios of the killing and *s*-mutation rates at the different r.h., relative to those at 97.5 per cent r.h., are compiled for 5 experimental

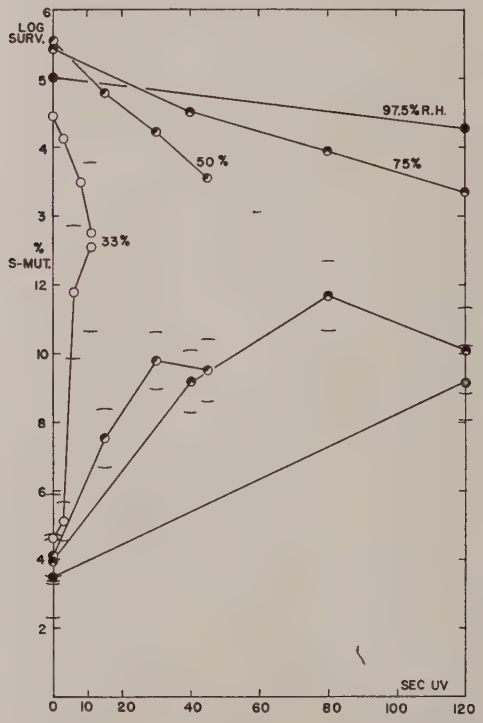


Fig. 1. Uv = dose curves of killing (upper part) and *s*-mutation (lower part) in bacteria at 97.5, 75, 50, and 33 per cent relative humidities (limits of error calculated from $\sqrt{p(1-p)/n}$).

series. It can be seen that the effectiveness of the uv on mutation and killing depends on the r.h. according to about the same function. The increase of uv sensitivity from wet (97 per cent r.h.) to dry (33 per cent r.h.) cells is more than 20 fold in both instances. This may indicate a similarity between the *induction process* of *s*-mutation and killing.

In a special series, uv irradiation was done at various times after putting dry cells (33 per cent r.h.) into the 97 per cent r.h. chamber. The results indicate that the full "protection" against uv by the water uptake was accomplished already after the shortest interval used, 13 min. Thus, the building up of the protecting action of the water is a rather fast process.

TABLE I

Dose-effect curves of survival and *s*-mutation in bacteria uv-irradiated in different relative humidities.

Exp. 26.10.54	Log no. survivors per 0.1 ml	Total no. of colonies counted	*w*-mutants		*s*-mutants		
			No.	%	No.	%	standard deviation, %
97.5 % r.h. (4.5 % NaCl)							
,, no uv	6.15	1276	15	1.17	18	1.41	±0.33
,, 60 sec. uv	5.75	1730	26	1.50	68	3.93	±0.47
,, 120 sec. ,,	5.13	1079	32	2.97	52	4.82	±0.68
,, 180 sec. ,,	4.75	1672	51	3.05	91	5.45	±0.55
75 % r.h. (sat. NaCl)							
no uv	6.36	1718	42	2.44	35	2.04	±0.34
20 sec. uv	5.81	1627	31	1.90	73	4.49	±0.53
40 sec. ,,	5.31	1834	57	3.11	110	6.00	±0.57
60 sec. ,,	5.04	—	—		—		
33 % r.h. (sat. $MgCl_2 \cdot 6\ H_2O$)							
no uv	5.61	1149	26	2.26	37	3.23	±0.52
3 sec. uv	5.23	1360	52	3.82	72	5.30	±0.61
6 sec. ,,	4.63	1131	34	3.00	80	7.05	±0.75
9 sec. ,,	4.51	2597	108	4.15	146	5.62	±0.45

To test whether the water state during uv irradiation alone determines the uv sensitivity or whether also the change in the water content *after* uv irradiation has an influence the following experiments were performed. Cells dried in 50 per cent r.h. were uv-irradiated, then one set was immediately exposed to 97.5 per cent r.h., while the other set was kept for ½ hour in the dry state and then moistened slowly in the stepwise fashion described above. A second double set of irradiated wet cells (97.5 per cent r.h.) was post-treated in a corresponding manner. The results in Table III show no different response of the killing to the two post-treatments in both double sets (except some additional killing independent of uv by the second drying in *w*/*d*). The *s*-mutation rates in both series where the bacteria were kept in the dry state and then slowly moistened after uv (*d*/*d* and *w*/*d*) seem to be a little smaller than in the series with a wet state immediately after uv (*d*/*w* and *w*/*w*). Comparing further the sets involving quick water changes (*d*/*w* and *w*/*d*) with those involving no (*w*/*w*) or slow (*d*/*d*) water change, we seem to see

Table II

Effectiveness of uv in bacteria and DNA irradiated at different relative humidities.

	Effect	Relative humidity (r.h.) 33 %	50 %	75 %	97.5 %
(*a*) *Serratia*					
Exp. series					
17.11.54	killing rate	27	6.0	2.3	1
11.11.54	,, ,,	—	3.8	1.8	1
3.11.54	,, ,,	22	—	—	1
26.10.54	,, ,,	19	—	2.7	1
21.10.54	,, ,,	28	—	—	1
Average	,, ,,	24	4.9	2.3	1
17.11.54	*s*-mutation rate	19	5.2	3.0	1
10.11.54	,, ,,	—	3.2	1.7	1
3.11.54	,, ,,	16	—	—	1
26.10.54	,, ,,	22	—	3.7	1
21.10.54	,, ,,	27	—	—	1
Average	,, ,,	21	4.2	2.8	1
(*b*) *DNA*	gel formation	22.1	3.7	—	1

a little lower *s*-mutation rate in the wet irradiated (w/d) and a little higher in the dry irradiated (d/w) series. But a pairwise dose to dose χ^2 test of d/d and d/w gives $\chi_4^2 = 5.38$ ($P = 0.14$); the same test for w/d and w/w gives $\chi_4^2 = 5.38$ ($P = 0.25$). Also the sum of both $\chi_8^2 = 11.81$ ($P = 0.16$) indicates no significant difference. On the other hand, the χ^2 test comparing dry and wet 30 sec. irradiated cells indicates a significant difference ($\chi_1^2 = 7.15$, $P = 0.0075$). Hence, the water content *during* uv irradiation determines mainly the uv sensitivity; but the water condition *after* irradiation has no significant influence, though some small differences within the limits of error cannot be excluded.

Several possibilities can be thought of *to explain the "protecting" action of water* against uv. (1) If uv acts indirectly in space by producing poison or mutagen molecules, e.g. fragments of nucleic acid, a simple *dilution* (decrease in concentration) by water may take place.[1] Using the formula of Zirkle and Tobias [27] or Six [25] for a simple indirect mechanism the

[1] The production of mutagenic organic peroxydes by uv as in uv-irradiated broth cannot play an important role in these experiments since the action spectrum of this process is much different from nucleic acid absorption (see Haas *et al.* [2]).

TABLE III

Influence of re-moistening procedure after uv irradiation.

Experiment	uv irradiation	Post-treatment	Log no. survivors	*s*-mutants	
				Ratio %	standard deviation, %
d/*w*	dry (50 % r.h.)	immediately into			
	0 sec.	97.5 % r.h. for 33 min.	6.18	12/910	=1.33±0.38 %
	10 sec.		5.86	46/1450	=3.17±0.46 %
	20 sec.		5.63	47/858	=5.29±0.77 %
	30 sec.		5.17	52/887	=5.86±0.80 %
d/*d*	dry (50 % r.h.)	after irradiation 33			
	0 sec.	min. at 50 % r.h.,	6.14	20/822	=2.44±0.54 %
	10 sec.	then 10 min. each at	5.66	35/915	=3.83±0.64 %
	20 sec.	60, 70, 80, 90 & 97 %	5.70	47/1017	=4.62±0.66 %
	30 sec.	r.h.	5.15	39/855	=4.52±0.71 %
w/*w*	wet (97.5 % r.h.)	after irradiation kept			
	0 sec.	at 97.5 % r.h. for 33	6.25	24/1436	=1.68±0.34 %
	30 sec.	min.	6.03	34/843	=4.04±0.68 %
	60 sec.		5.70	68/1385	=4.91±0.58 %
	120 sec.		5.23	84/1347	=6.22±0.66 %
w/*d*	wet (97.5 % r.h.)	same as *d*/*d*, resulting			
	0 sec.	in quick drying and	5.84	16/615	=2.61±0.64 %
	30 sec.	slow remoistening	5.88	47/1466	=3.21±0.47 %
	60 sec.	after uv	5.38	79/1794	=4.41±0.49 %
	120 sec.		5.02	38/801	=4.47±0.75 %

effectiveness of the uv would be $k = 4\pi RDTc\,(1 + R/\sqrt{DT})$, where c is the concentration of the diffusing active molecules, T their mean lifetime, D their diffusion constant, R the radius of the sensitive spot which must be reached by a molecule in order to continue the reaction chain leading to the effect. The concentration c may be inversely proportional to the r.h., since the water uptake is probably mainly osmotic. Then a function $k \sim 1/\text{r.h.}$ is to be expected and the increase of k from 97.5 per cent r.h. to 33 per cent r.h. should be from about 1 to 3. Actually, more than 20-fold increases were obtained (Table II), which does not fit this simple hypothesis. The high increase found seems to indicate some additional, more active protection by the water.

(2) The water itself, the O_2 dissolved in it, or some metabolic system acti-

vated by the water may interfere chemically with the supposed "poison". This means, in terms of the above model, a decrease in the lifetime, T. In the case of $R \ll \sqrt{DT}$ (where $\sqrt{DT}$ is the mean action radius of the poison), since dilution would also act here, we get $k \sim 1/(\text{r.h.})^2$; e.g. k (97.5 per cent)/k (33 per cent) $\sim 1/9$. This fits the results much better, and would agree even more if we assume a decrease of T more than proportional to the r.h., as if an adsorption mechanism for the interfering molecules were involved. Nevertheless, dissolved O_2 cannot be the protector, since earlier studies revealed no changed killing, and even a slight increase of the *s*-mutations, when uv irradiation was conducted in air as compared with N_2 [13]. Since drying destroys the protection and resoaking restores it very quickly, a supposed protecting metabolite would have to be destroyed by drying, and produced very fast during re-moistening. How water itself could be the inactivating chemical is not easy to imagine. Thus, this second group of explanations is not promising.

(3) There remains the possibility that the water may directly change the structure of the target, the nucleic acid, so that the quantum efficiency for starting the bio-reactions is lowered by incorporation of water. That this may be the true explanation is suggested by the formation of an insoluble gel from DNA if the latter is uv-irradiated as a dry film, but not if it is irradiated in solution [23]. This fact led us to investigate this gel formation by films of DNA uv-irradiated in states of different water content [18]. Results obtained by the described methods for grading the moisture content show that indeed gel formation decreases with the r.h. in a manner similar to the biological responses of the bacteria (Table IIb).

So the gelation is probably initiated by a similar chemical reaction in the DNA as are the reactions leading to *s*-mutation or killing. The essential first step for all three effects may be the breaking of H-brigdes by uv. In the case of gelation new H-bridges may then be formed at the contact points between the DNA fiber-molecules, in the case of mutation (or killing) perhaps other steps may follow, e.g. tautomeric changes in pairs of aromatic bases which finally cause "copy errors" (or more serious damages = killing) during the reproduction of the DNA chain. In the dry state the quantum efficiency of these processes is very high compared with other changes observed in DNA, e.g. viscosity decrease, because doses of small biological effectivity (200 ergs/mm^2) give remarkable gelation (see also [24]). Our results suggest that a structural change of the DNA *in vitro* and *in vivo* occurs between about 30 and 50 per cent r.h. which strongly decreases the uv sensitivity (energy transfer from site of absorption to site of reaction?) in the hydrated state.

Propably this structural change is the same as the one relating water structure to the coiling state of DNA [4].

Since this DNA change is able to account fully for the observed change in the uv sensitivity of the cell, it appears improbable that diffusion of "poisons" (indirectness in space) plays an important role in uv killing and *s*-mutation; for when a dilution effect, as described in (1), is superimposed, there should be an increase of uv sensitivity from 97.5 to 33 per cent r.h. much greater than the 20-fold one found.

Water content and photoreversion.—For the study of the effect of post-treatment with light only 33 per cent (dry) and 97.5 per cent (wet) r.h. were used. In Table IV the results of one of the series of experiments are shown where *dry cells* were *uv- irradiated.* No influence on the slope of the killing curve can be seen due to the illumination in either the wet or dry state. The same is true for the *s*-mutants, which increase about 2 per cent after 5 sec. uv despite the posttreatment. (The *w*-mutants show no clear response to uv). So both uv effects are not photoreversible when dry cells are uv-irradiated.

TABLE IV

Post-treatment of dry uv-irradiated bacteria with light.

	Post-treatment	Log no. survivors	Total no. of colonies	*w*-mutants		*s*-mutants		
				No.	%	No.	%	Standard deviation, %
(*a*) *uv. at 33 % r.h.*								
no	no	5.23	1023	24	2.34	23	2.24	±0.46
5 sec.	light	4.52	662	14	2.12	30	4.52	±0.81
10 sec.		3.89	—	—		—		
no	1 h. light at	4.82	1562	17	1.09	30	1.92	±1.10
5 sec.	97.5 % r.g.	4.05	787	18	2.29	31	3.94	±0.70
10 sec.		3.52	668	11	1.69	34	5.09	±0.85
no	1 h. light at	2.82	598	22	3.68	27	4.51	±0.85
5 sec.	33 % r.h.	2.25	321	11	3.42	22	6.85	±1.40
10 sec.		1.64	142	5	3.51	5	3.51	±1.55
(*b*) *Undried controls, always at 97.5 %* r.h., *no uv*								
	dark	6.06	883	29	3.29	13	1.47	±0.41
	1 h. light	6.16	1070	38	3.55	23	2.15	±0.44

On the other hand, light alone kills these cells, even more when dry cells are illuminated. It also induces *s*-mutations in them ($t=2.32$, $P=0.019$, for no uv, no light against no uv, 1 hour light). But this light action is restricted to cells that had been dried some time, since cells kept wet are not influenced by the light dose used her ($t=2.47$, $P=0.012$, for 1 hour light, no uv, dry against undried. Table IVb). Hence, drying, which alone kills about 70 to 80 per cent of the cells, causes photosensitivity.

As shown by Table V the light response of cells *uv-irradiated in the wet state* (equilibrated at 97.5 per cent r.h. after drying on the film) is different. Group (*b*) indicates a clear photoreactivation of "killed" cells and a rever-

TABLE V

Post-treatment with visible light of wet uv-irradiated bacteria in the dry (*a*) and wet (*b*) state. (Cells were equilibrated at 97.5 per cent r.h. for 2 hours, in (*a*) uv.-irradiated for 0 or 120 sec.; then dried quickly at 33 per cent r.h. and illuminated for 0, 15, 30, or 45 min.)

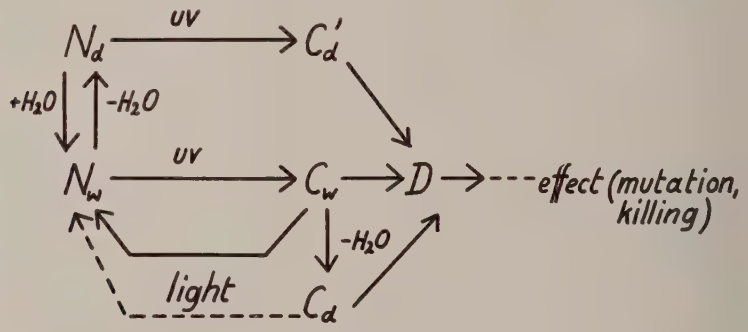
(*a*) uv (97.5 % r.h.)	Light (33 % r.h.)	Log no. survivors	Δ log no. survivors	*s*-mutants		
				Ratio	%	Standard deviation, %
0 sec.	no	5.45	0	16/857	=1.87	±0.46
120 sec.	no	4.03	0	65/860	=7.57	±0.90
difference	0–120 sec. uv	1.42			5.70	±1.10
0 sec.	15 min.	5.20	−0.25	20/1153	=1.74	±0.38
120 sec.	15 min.	3.33	−0.70	40/609	=6.59	±1.01
difference	0–120 sec. uv	1.87			4.85	±1.07
0 sec.	30 min.	4.66	−0.79	25/1109	=2.25	±0.43
120 sec.	30 min.	2.99	−1.04	49/870	=5.63	±0.78
difference	0–120 sec. uv	1.67			3.38	±0.89
0 sec.	45 min.	4.06	−1.39	17/1539	=1.10	±0.27
120 sec.	45 min.	2.64	−1.37	49/1255	=3.90	±0.55
difference	0–120 sec. uv	1.42			2.80	±0.61
(*b*)	Light (97.5 % r.h.)					
0 sec.	60 min.	4.52		32/915	=3.49	±0.62
120 sec.	60 min.	4.41		38/824	=4.61	±0.74
240 sec.	60 min.	3.79		54/901	=5.98	±0.79

sion of the *s*-mutations when light was given in the wet state. The uv dose is reduced by one hour light to about 1/5 for killing and to 1/9.5 for *s*-mutation (see also [16]). But if dry cells were exposed to light no reactivation occurred, as is shown by about the same differences in log survivors after 0 and 120 sec. uv irradiation at the different light doses. Nevertheless, the *s*-mutations are reversed in these dry illuminated cells as is indicated by the decreasing differences with increasing light dose between mutant percentages after the 0 and 120 sec. uv doses. This reversion is significant since the *t*-test between these differences at 0 and 45 min light dose gives $t = 2.32$, $P = 0.019$, and, further, since there is a clear seriation of these differences with the light dose, which can be expected by chance only in $1/4! = 1/24 = 0.042$ cases. Reversion is also found in two other experiments where the differences, taken singly, lie within the error limits.

Table V shows again *effects of the light alone*. Clearly the bacteria are killed when illuminated in the dry state (see above). This killing seems to be a single hit process and its rate is about the same in uv-irradiated and unirradiated cells (see Δ log survivors). Thus, this killing by light is apparently independent of the killing by uv. In contrast to the killing and *s*-mutation induction already found (Table IV) after wet-illumination when the cells were previously dried (which can be seen again by comparing the 0 sec. uv effects of group (*a*) and (*b*) of Table V), this killing by dry illumination does *not* parallel *s*-induction (compare the 0 sec. uv effects in the different light doses). Both effects of light alone (killing and *s*-mutation) seem to depend on the dehydration state before and during the illumination in a complicated manner. Apparently the duration of the drying period before and during illumination plays a role, since this period was about 1 h in experiments (Table V) where no induction of *s*-mutations by light was observed, but it was about 3 h in experiments (Table IV) where *s*-mutations have been induced. The cause of the photosensitization by drying has still to be cleared up in further studies. Perhaps the water-insoluble red pigment typical for *Serratia* is changed into a "photodynamic" state, or other light-absorbing compunds are produced during drying. On the other hand, the compounds or structural states responsible for photoreactivation seem to be inactivated by drying. So the photoreversion and photoreactivation after uv irradiation appear to be relatively independant of the killing and *s*-mutation by light. This is supported also by the fact that photoreversion was found in cells which were always kept wet [16], but the latter processes were observed (with the light doses used here) only in dry or predried bacteria. The induction of *s*-mutations and killing by light responded differently to drying;

this indicates a different though similar nature of both processes as it is shown also by the photoreversion of both uv-induced effects.

The observations on *photoreversion* are summarized as follows: If dry cells are uv-irradiated, there is no reversion at all; if wet cells are uv-irradiated there is reversion of both effects by light given on wet cells, but reversion of *s*-mutations only, not of killing, when light is given on dry cells. These facts can be pictured by a system of reaction steps between different states which are induced by the external factors:



N_w, N_d, C_w, C_d, C_d', D symbolize states of the cell or its reacting material, of which letters with the suffix w are the wet states, with d the dry ones. The arrows mean turn-over reactions leading finally to the observed effects, killing or mutation, or the normal states, N_w, N_d. The factors producing the turn-overs, uv, light, and water, are indicated beside the arrows. The normal wet state N_w is converted by uv into the photoreversible (colored) state, C_w. If C_w is turned by drying into C_d it has a reversible pathway (dotted line) only in the case of *s*-mutation, not of killing. The normal dry state, N_d, is turned by uv to a different state, C_d', that is not photoreversible, but passes into an irreversible state D leading to the effects studied. Of course, two different systems or substances (e.g. different DNA's, or DNA and RNA) are to be assumed for these reactions, one for *s*-mutation, one for killing. At least four premutational and pre-killing states are revealed by these observations. They may be structural types of the same substances, such as nucleoprotein, or consist of cooperation between different substances.

A simple model of chemical cooperation may be obtained assuming that photoreversion involves the production of an inhibitor substance by the illumination of a colored precursor. The inhibitor would, by reaction with the uv-induced state or substance C, turn the latter to its normal state N.

The precursor or inhibitor must be assumed to be different for killing and mutation, being unstable against drying for the first and stable for the second effect. In dry cells the substance N is changed by uv into C or directly to D which are not attacked by the inhibitor. The findings of Hill [3] with phages indicate a similar photo-irreversible state (C_d') produced by uv irradiation of dry phage. But no changed uv sensitivity of the dry phage was found. Higher sensitivity was obtained first by very strong drying, e.g. in alcohol. Thus, the more sensitive (coiled?) state of phage-DNA seems to be obtained at lower r.h. than is required in *Serratia;* but the photo-irreversible state is reached already by less dehydration. Since the photoreversion at only two r.h.'s was studied in *Serratia* it is possible that here, too, a photo-irreversible, but still more uv-insensitive, state exists at an intermediate humidity range.

In general, the findings reported support the view that the structure and radiosensitivity of the target substance in the cell can be influenced strongly by external factors and that such influences need not to be explained by the assumption of spacially indirect action of the radiation.

SUMMARY

Resting cells of *Serratia* were spread on a dry film of gelatin on a glass slide, dried, brought to different water contents in quartz covered chambers with salt solutions giving from 33 to 97.5 per cent relative humidities (r.h.), and then uv-irradiated. After dissolving the gelatin to suspend the cells, diluting, and plating, survivors and color-sector (*s*) mutants were counted. The main facts on the nature of *s*-mutations are reviewed.

The relative uv sensitivities, measured as the slopes of the one-hit curves for killing and mutation, decreased from more than 20 to 1 when the cells were moved from 33 to 97.5 per cent r.h. and this decrease was about the same for mutation and killing. The decrease in radiosensitivity was accomplished in less than 13 min. The water content after irradiation seemed to play no appreciable role. The "protection" by water against uv cannot be due to simple dilution of supposed uv poisons, but it goes parallel with a change in the structure of the uv-absorbing DNA brought about by moisture content, which can be observed *in vitro* as a decrease of uv-induced gel formation by DNA. No indication of spacially "indirect" (diffusion) processes in the uv action was found.

When dry (33 per cent r.h.) cells are uv-irradiated no photoreversion of either killing or mutation is observed with either dry or wet (97.5 per cent r.h.) illuminated cells. Wet uv-irradiated cells give photoreactivation of killing

only when wet cells are illuminated, but *s*-mutations are photoreversed when wet or dry cells are exposed to light. A hypothetical reaction system which explains these effects is discussed.

When cells are illuminated in the dry state, visible light alone induced killing but not *s*-mutation. This killing seems to be a one-hit process and its rate is the same in previously uv-irradiated and unirradiated cells. Light alone did not cause killing or mutation in cells kept in wet state; but cells previously dried and then illuminated when wet showed some killing and *s*-mutation induction by light alone.

The authors are indebted to Dr. Francis J. Ryan for his generous invitation to continue work on *Serratia* in his laboratory, and for fruitful discussions. The work was supported by a Boese Postdoctoral Fellowship, a Fulbright Travel Grant, and in part by an American Cancer Society Grant recommended by the Committee on Growth, by a Research Grant from the National Institutes of Health, U.S. Public Health Service, and by Contract No. AT(30-1)-1734 with the Atomic Energy Commission.

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ELECTRON MICROSCOPY OF MAST CELLS IN THE SKIN OF YOUNG MICE

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During an investigation of the fine structure of developing hair follicles and of growing hairs [17], cells containing granules in their cytoplasm were seen in electron micrographs of the dermis of 3-day-old mice. At this stage the dermis is highly cellular but the cells described here could readily be distinguished by their electron-dense granules. These are approximately 0.5 μ in diameter.

The granules are present in the cytoplasm together with mitochondria, a small vesicular component, and the closely packed agranular membranes of the Golgi apparatus.

In view of the fact that an active synthesis of collagen takes place in the developing dermis it was thought that the granule-containing cells might be related to the fibroblasts described recently by Jackson [9, 10] and by others [16]. However, subsidiary phase contrast microscopy and histochemical tests have shown that the cells are mast cells. Since detailed information on the fine cytology of these cells at the electron microscope level is lacking, the results are reported here.

METHODS

Small pieces of dorsal skin were rapidly removed after decapitation of 3-day-old mice. The pieces (2 mm × 2 mm) were fixed for $3^1/_2$ hours in 1 per cent osmium tetroxide buffered to pH 7.4 [11] at room temperature. They were washed in Ringer for 30 mins, dehydrated, and finally embedded in *n*-butyl methacrylate containing 5 per cent of the methyl monomer. Specimens were polymerized at 45°C.

Thin sections were cut on a modification of the microtome described by Hodge *et al.* [7] and mounted on carbon films on copper grids. They were examined in a Siemens Elmiskop I electron microscope at 80 KV with a 25 μ objective aperture. Thicker sections also were cut and collected on small pieces of coverslip, mounted in mineral oil and examined by phase-contrast microscopy.

For histochemical tests, the skin was fixed in Carnoy for 6 hrs, paraffin embedded and sectioned at 8 μ. For Periodic acid-Schiff (PAS)-positive substances

the method of Hotchkiss [8] was utilised, the action of saliva for 30 min serving to eliminate glycogen. Basophilic metachromasia was studied after staining in 0.5 per cent toluidine blue, 0.05 per cent thionin, and *M*/2000 methylene blue at pH 1.5 (N/20 HCl). The stained sections were examined mounted in water, and permanent mounts were prepared by dehydration in tertiary butyl alcohol followed by mounting in DPX.

RESULTS

Fig. 1 is an electron micrograph of a region of the presumptive dermis in the skin of a 3-day-old mouse. The epidermal cells of the primordium of the hair follicle (*B*) are surrounded by a basement membrane that develops into the glassy layer [17] and a layer of closely-linked fibroblasts (*F*). Near the follicle can be seen a mast cell (*M*) with an irregular boundary and containing granules. Two other cells (*D* and *E*) of smoother outline with mitochondria and occasional profiles of endoplasmic reticulum in their cytoplasm, have no granules and are probably fibroblasts. Intercellular areas of small bundles of young collagen fibrils (*C*) can be distinguished.

The mast cells are scattered among the mesenchymal cells of the young dermis. They are found in the position shown in Fig. 1, but do not concentrate at any particular follicle level. They are progressively more difficult to find in sections from older animals. A further location is shown in Fig. 2, where a Schwann cell containing numerous unmyelinated axons has a mast cell fibroblast investment. Young collagen fibrils are situated in the space between the cell membranes of mast cell, fibroblast, and Schwann cell.

The finer details of the cytoplasm of a mast cell are revealed at higher magnification (Fig. 3). The mast granules are markedly electron dense, and irregular in shape. They vary from 0.3 μ to 1 μ in diameter but are usually about 0.5 μ wide. The periphery of a granule is denser than the interior. The number seen in the cytoplasm of a section through a mast cell varies considerably and doubtless depends on the plane of section as well as on actual concentration of granules which is known to differ considerably from cell to cell. There is usually a cytoplasmic space surrounding each granule but no limiting membrane lining the space has been observed. Mast granules have not been found extra-cellularly. Spaces with no granules appear in the cytoplasm and since granules are found predominantly at the cell periphery, it is possible that they are extruded from the cell.

Electron micrographs have shown that the interior of the granules has the appearance of a network of filamentous elements (Fig. 4). However higher resolution studies are required to investigate this point, since the dimensions of the filaments are near the limits of resolution in the present study.

Apart from the granules, the cytoplasm of the mast cells contains many other organelles. A consistent feature is a well-organized system of closely packed membranes in an oval configuration situated near the nucleus (*G*, Fig. 3). These membranes correspond to the agranular reticulum of Palay and Palade [15] and are similar to the lamellar component of the Golgi apparatus as described by Dalton and Felix [5] and others [13, 18].

Mitochondria of both rounded and rod-shaped cross-sectional outline are observed in sections of mast cells. They vary in size, up to about 0.2 μ in diameter and 1 μ long, and possess recognizable internal cristae as described for many animal cells [12].

The remainder of the cytoplasm is dense and contains numerous small circular profiles 400–500 Å in diameter which are concentrated in the general region of the Golgi membranes. Indeed it has been noted in the cells examined so far that the granules tend to accumulate at the cell poles and periphery, and a zone near the nucleus is free of granules but contains other cell organelles, such as the mitochondria, Golgi membranes and vesicles. Very little, if any, endoplasmic reticulum with attached cytoplasmic particles is observed in the cytoplasm of mast cells whereas considerably more is found in fibroblasts.

The identification of the cells here described as mast cells has been established by direct comparison in the phase contrast microscope of the cells in relatively thicker methacrylate sections cut from the same block which was used for electron microscopy. Histochemical tests have been applied to paraffin sections of skin of the same age.

A mast cell can be seen in a phase-contrast micrograph of an area in the developing dermis (Fig. 5). The cell is situated between two hair follicles. The cytoplasmic space surrounding the mast granules is evident. The cells are generally oval often with an eccentric nucleus.

In paraffin sections stained by the PAS method the same cells can again be identified by their morphology and distribution; granules of similar dimensions to those described above stained with moderate intensity (Fig. 6). The staining intensity was slightly reduced by prior digestion of the sections with ptyalin. The mast granules were markedly metachromatic after staining with toluidine blue, thionin (Fig. 7) or methylene blue; metachromatic basophilia was still displayed after staining with methylene blue at pH 1.5. The PAS and metachromatic staining described here, agree with the staining properties described by Compton [4] and Asboe-Hansen [1] for mast cells and attributed to the presence of sulphated mucopolysaccharides of high molecular weight in the granules.

DISCUSSION

The scarcity of collagen fibrils in the skin of newly-born mice enables sections to be obtained which are thin enough for electron microscopy. Adult skin is much more difficult to section for this purpose.

The mast cells studied have a distinctive cytoplasm and their identity in electron micrographs has been established by reference to features observable in the light microscope.

The cytoplasmic granules described by Jackson [9, 10] from electron microscope studies of fibroblasts, osteoblasts, and osteocytes, in the embryo and cultivated *in vitro* are similar in some respects to the granules of mast cells. In fibroblasts, according to Jackson, the granules are about 0.7 μ in diameter with a dense periphery and are associated with dense lamellae. Histochemically they are PAS positive and are also metachromatic when stained with basic dyes, but the metachromasia is destroyed by fixation and treatment with alcohol. The metachromasia of mast granules is, however, much more resistant.

In the present work, fibroblasts with cytoplasmic granules as described by Jackson and others, have not been observed either by phase microscopy or electron microscopy and this may be correlated to the small amount of collagen fibrils present at the stages investigated. However, both cell types are associated in some unknown way with connective tissue development and maintenance. Mast cells may be responsible for the laying down of components of the "ground substance" in regions where the synthesis of collagen takes place. The secretion of mucopolysaccharides is among their many possible functions, for according to Asboe-Hansen [1], it seems likely that they secrete both mucopolysaccharides of the hyaluronic acid type and sulphated derivatives—the "heparin-like" substances. However, it should be noted that this interpretation has not received universal acceptance [2, 6].

The structural features here described in the cytoplasm of mast cells are consistent with the appearances to be expected in a cell actively engaged in secretion. The abundant granules in such cells obscure any mitochondria stainable by supra-vital techniques, and the presence of mitochondria, therefore, has remained in doubt [4]. The electron microscope, however, has

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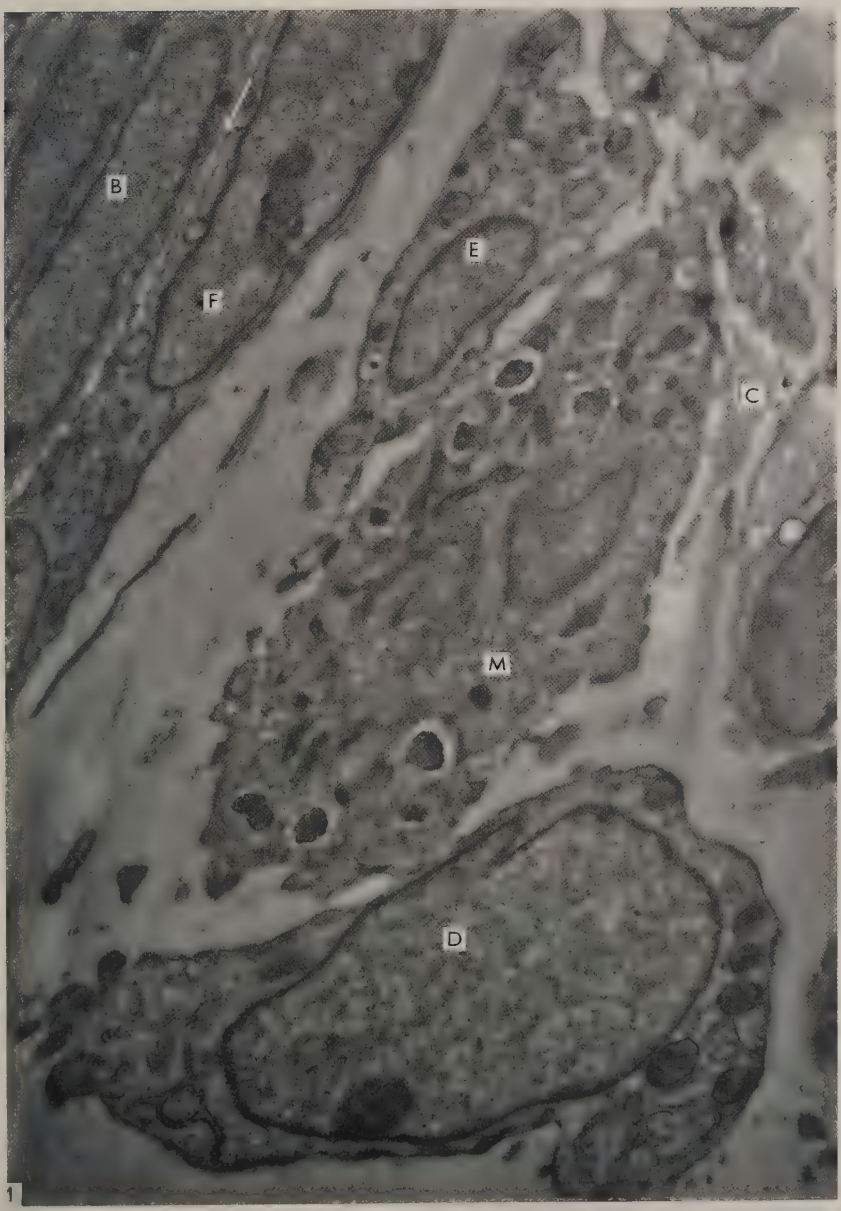
Fig. 1. An electron micrograph of the developing dermis of a 3-day-old mouse. *M*, mast cell; *B*, hair follicle; *D*, *E* and *F*, fibroblasts; *C*, collagen. The glassy layer (arrow) is located between the hair follicle (*B*) and the fibroblast layer (*F*). ×15,000.


B
E
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1

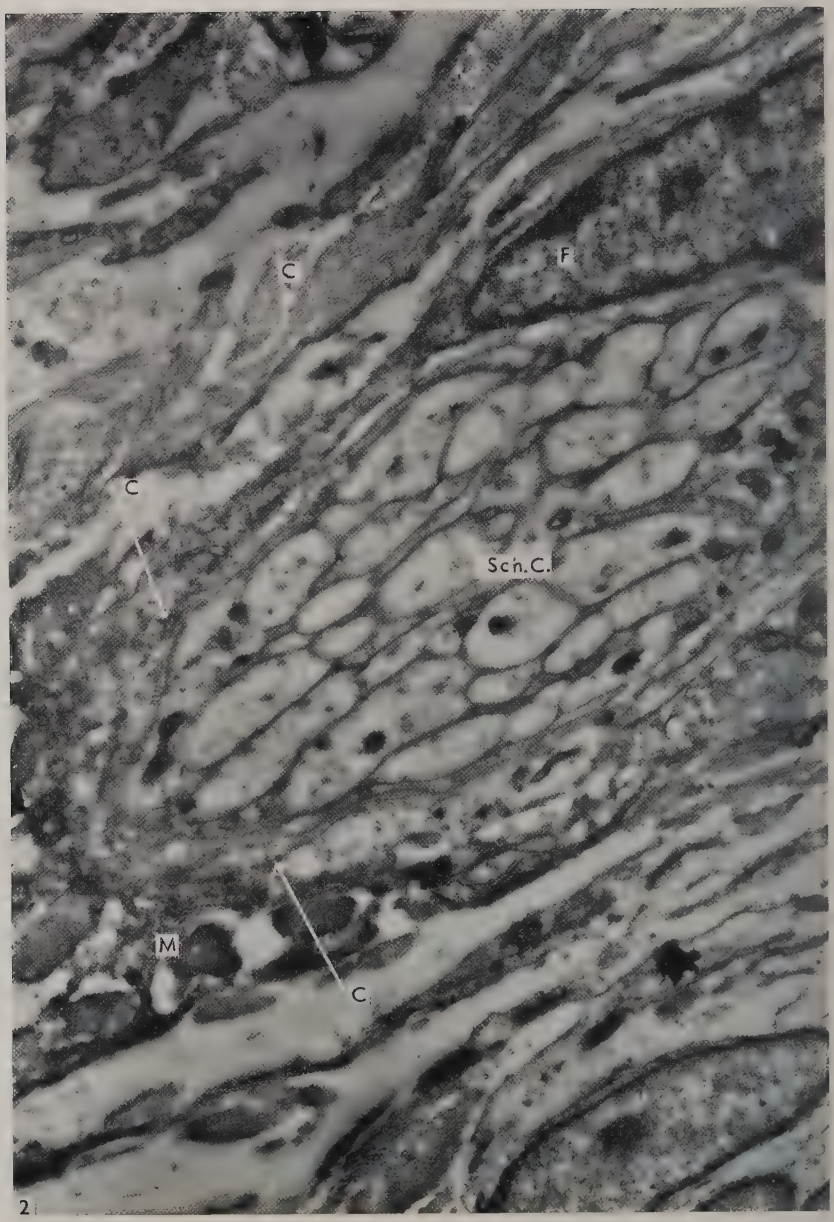


Fig. 2. An electron micrograph of a Schwann cell (*Sch. C.*) surrounded by a mast cell (*M*) and fibroblast (*F*). Collagen fibrils (*C*) are present between the cells (arrows). × 15,000.

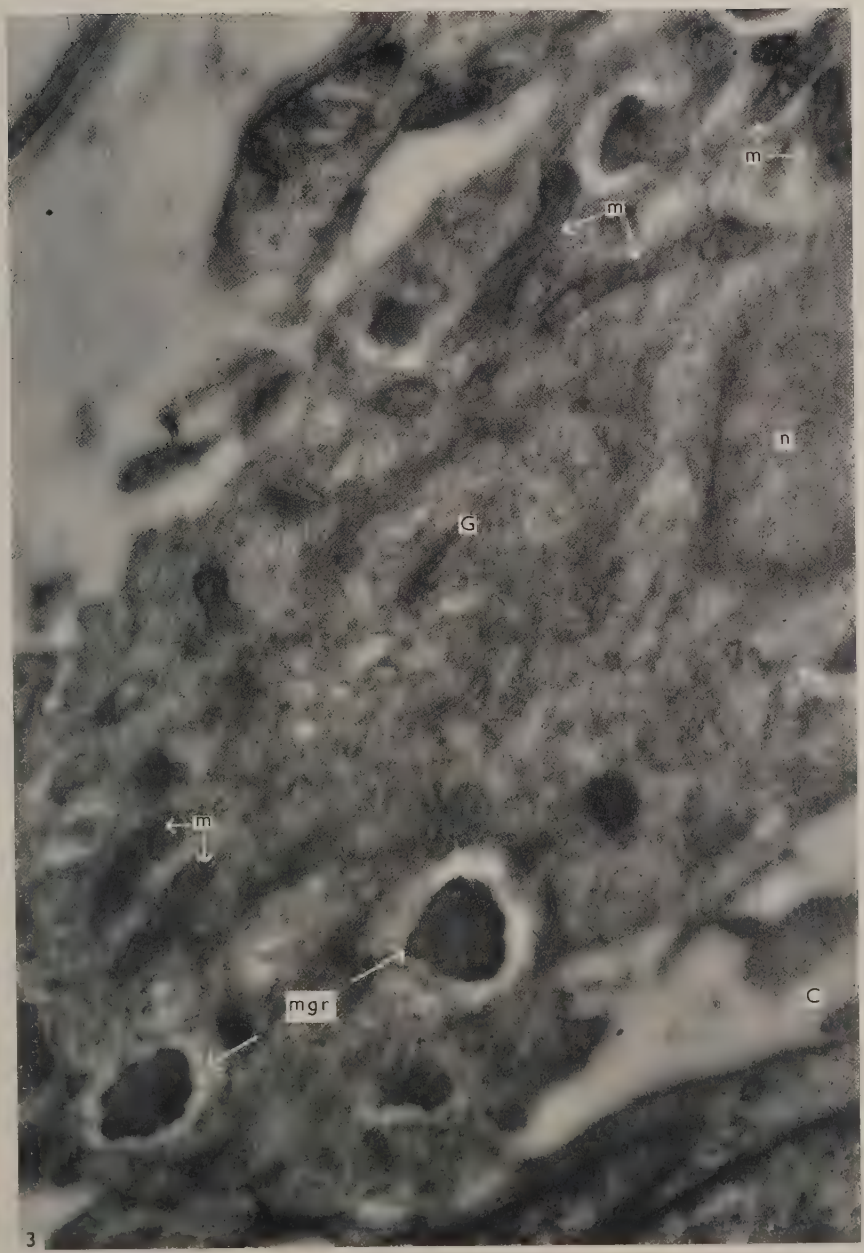
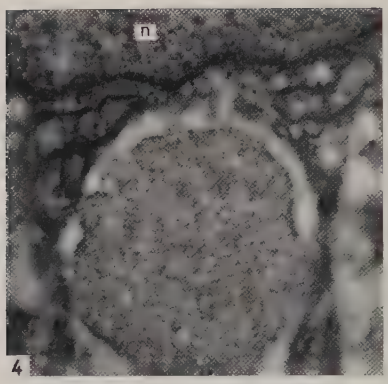
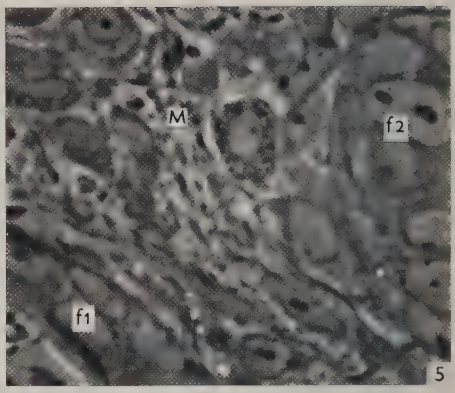
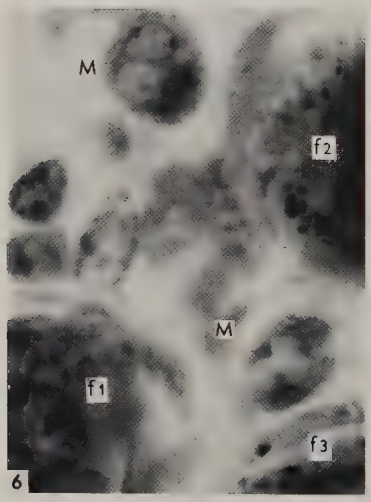


Fig. 3. An electron micrograph of a mast cell showing cytoplasmic components. *m*, mitochondria; *m. gr.*, mast cell granules; *G*, Golgi zone; *n*, nucleus. · 30,000.







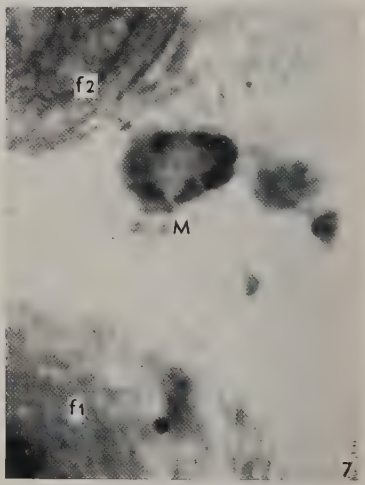


Fig. 4. A section through a mast cell granule showing evidence of a filamentous internal structure. ×40,000.
Fig. 5. A phase contrast of a mast cell situated between hair follicles (f_1, f_2) in the dermis. ×1900.
Fig. 6. PAS-positive granules in a mast cell located near hair follicles (f_1, f_2, f_3). Glycogen granules are present in the outer root sheaths of the hair follicles. Paraffin section. ×1900.
Fig. 7. Metachromatic granules in a mast cell located between hair follicles (f_1, f_2). Paraffin section, thionin. ×1900.

shown that typical spherical and rod-shaped mitochondria are present in the mast cells studied. Compton [4], using staining methods, found only occasional evidence of a Golgi apparatus in mast cells, but in electron micrographs the closely packed membranes of this organelle form a relatively large system near the nucleus. Occasional slight dilation of the membranes occur but no large vacuolar component is associated with them. The numerous small vesicular structures 400–500 Å in diameter, are of interest; similar circular profiles seen in other cells have been included in the Golgi complex [3, 19]. The cytoplasm of mast cells has been described as basophilic [4], but so far little or no endoplasmic reticulum associated with cytoplasmic particles of 100–200 Å diameter has been observed. It is concluded that the cytoplasmic particles, which, according to Palade [14] contain RNA, are for the most part free in the cytoplasm of mast cells.

Mast granules have previously been described as generally globular in shape, but after osmic fixation many are irregular in appearance. Membranes limiting the cytoplasmic spaces in which the granules are situated have not been observed but the surface of the granules is dense. The filamentous texture of the mast granule suggests that the constituent mucopolysaccharides of high molecular weight have some submicroscopic organization.

SUMMARY

Mast cells have been identified in the dermis of 3-day-old mice, and studied in the electron microscope.

The metachromatic granules are electron dense bodies which appear to be made up of filamentous material. Each granule has a dense periphery and is generally surrounded by a cytoplasmic space. In addition to the granules the cytoplasm contains spherical and rod-like mitochondria of typical internal structure. Closely-packed Golgiform membranes are present in the cytoplasm together with small vesicular components but there is little or no endoplasmic reticulum contrasting with the situation found in the cytoplasm of fibroblasts.

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ALKALINE PHOSPHATASE AND THE CALCIFICATION IN TISSUE CULTURES

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The role of alkaline phosphatase in calcification has been a subject of lively discussion ever since Robison [16] in 1923 demonstrated alkaline phosphatase in bone tissue and in calcifying cartilage. In the beginning it was thought that the mechanism of calcification could be explained by enzymatic splitting of organic phosphates, followed by precipitation of calcium phosphate when the solubility product of calcium phosphate was exceeded.

However, it was soon found that this explanation was not satisfactory. Tissues were found which had a large content of alkaline phosphatase though normally they did not calcify. This was the case with kidney tissue. Further Fell and Robison [6] found that calcification does not take place in embryonic avian cartilage although this, as well as mammalian cartilage, has a high content of alkaline phosphatase in the zone of hypertrophied cartilage cells. In such cases Robison assumed the alkaline phosphatase to produce a rise in the concentration of phosphate ions. He introduced the idea of "a second mechanism" favouring the deposition of calcium phosphate from supersaturated solutions, this mechanism being a property of tissues which calcify [17]. Other tissues lacking this second mechanism do not calcify, even if they contain alkaline phosphatase. Robison says: "*If* the enzyme in these other tissues sets free inorganic phosphate in a fluid already saturated with calcium salt, why is this salt not deposited in these tissues as it is in bone?" [18]. Many investigations concerning this "second mechanism" are based on the assumption that inorganic phosphate *is* set free as a result of the action of the phosphatase where this enzyme occurs. The objective of the above-mentioned workers has therefore been to follow the process of calcification in tissues possessing the "second mechanism" without being calcified. Thus, hypertrophied cartilage from rachitic mammalian epiphyses has been used for such experiments *in vitro*, and a study has been made of the calcification taking place after the tissue was brought in contact with various supersaturated solutions of calcium and phosphate.

The numerous investigations made into the physiological action of alkaline phosphatase and the problem of calcification in inorganic solutions have thrown much light on the process of calcification. It seems that phosphatase is active only during the first stage of the formation of the complex apatite crystal, the complete development involving many still unexplained chemical and physical mechanisms.

The alkaline phosphatase in non-calcifying tissues is certainly involved in metabolic processes which normally have nothing to do with calcification. In tissues which calcify, it is assumed that the phosphatase is related to the pre-osseous cellular metabolism such as in the formation of the bone matrix [20]. These investigations have not solved the problem of "the second mechanism", and we still have no explanation why there is no calcification in embryonal avian cartilage or in normal kidney tissue.

A relatively simple living tissue such as a tissue culture could be expected to yield valuable information on the problem of calcification; and it is surprising that the relationship between alkaline phosphatase and calcification in tissue cultures has never been investigated. The present paper may give some contribution to fill this gap.

The precipitation of calcium phosphate in cultures of chicken heart fibroblasts cultivated in the presence of organic phosphates has recently been described [1]. A formation of alkaline phosphatase during cell degeneration in cultivated heart fibroblasts was also observed [10]. In this paper the relationship between alkaline phosphatase, calcification and cellular alterations (degeneration, cell death) in tissue cultures will be described. Based on these observations, an explanation of the initial process of calcification is offered.

EXPERIMENTAL

Materials and methods.—Slide cultures of heart fibroblasts from 9-day-old chicken embryos were used when not otherwise stated. The medium consisted of one drop of chicken plasma and one drop of embryo extract (crude embryo extract diluted 1:1 with Tyrode's solution). The cultures were normally transferred to new media every 2–3 days. The middle piece with the original explant was preserved in some of the cultures. Heart fibroblasts were used after cultivation on coverslips for 5–6 passages. The cultures were halved and transferred to Carrel flasks. The solid phase consisted of 0.5 ml chick plasma and 1.0 ml Ringer's solution clotted with one drop of embryo extract. The fluid phase consisted of 0.4 ml serum, 0.1 ml Tyrode's solution and 0.1 ml of a solution of calcium-β-glycerophosphate (60 mg dissolved in 20 ml 0.9 per cent NaCl). In the control cultures 0.2 ml Ringer's solution was used instead of the glycerophosphate solution. Cultures of heart fibroblasts grown on perforated cellophane as described by Evans and Earle [5] in a fluid medium composed of 0.6 ml

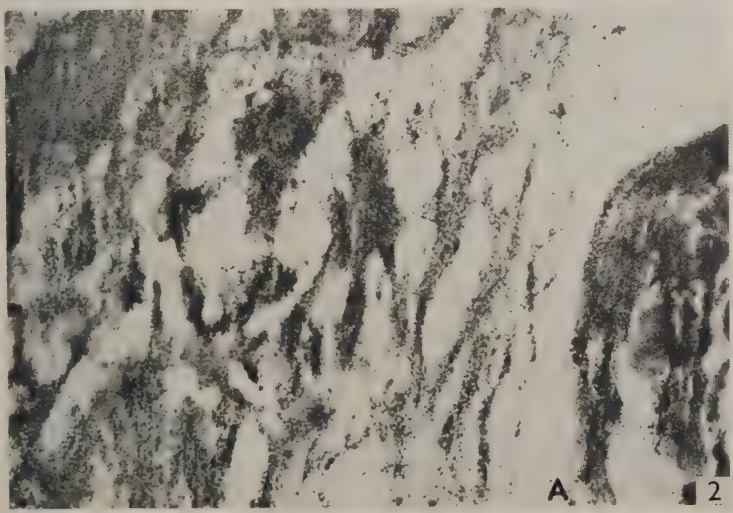


Fig. 1. Section of fibroblast culture, 4th passage. Normal media. Von Kossa staining for calcium phosphate deposits. Note the localization of the calcification in the areas inside the cut edges. The calcification is most extensive in the periphery of the migration zones, along the cut edges. Magnification × 40.

Fig. 2. Section of fibroblast culture, 4th passage. Normal media. Von Kossa staining. Note the cellular limitation of the calcification in the more central areas of the calcification, which is extended nearly until the cut edge of the precedent passage (*A*). Here and in the periphery the calcification is more solid, including the intercellular spaces. Magnification × 475.

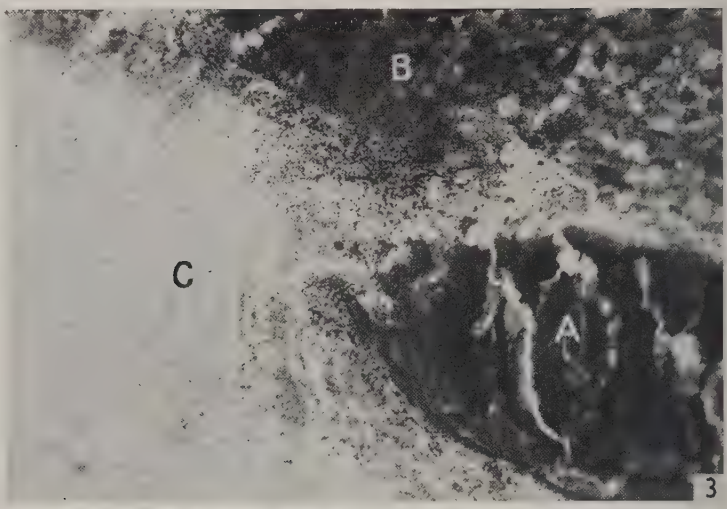


Fig. 3. Section of fibroblast culture, 3rd passage. Normal media. *A*, Calcified area of the second passage; *B*, calcified area of the third passage; *C*, uncalcified area of the third passage. Magnification × 285.

Fig. 4. Section of the same culture as Fig. 3. Manheimer-Seligman staining for alkaline phosphatase. Note that only a few cells in the calcified areas stain for alkaline phosphatase. Most of the phosphatase-stained cells are located in the uncalcified area *C*.

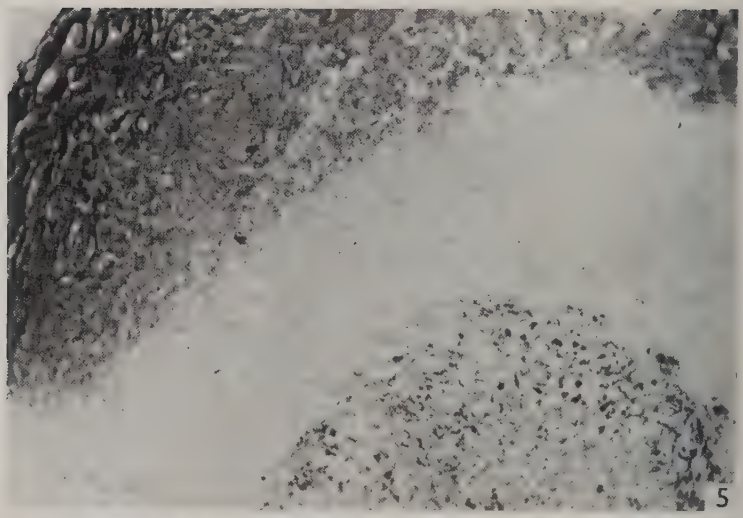
chick serum, 1.2 ml Tyrode's solution, 0.2 ml embryo extract and 0.1 ml of the above-mentioned glycerophosphate solution were used for special purposes.

The cultures were washed in Ringer's solution for 5 min. and then fixed in 80 per cent alcohol for 30 min. Cultures which were prepared as whole mounts were taken down to water, stained and mounted. Serial sections were performed as described by Enghusen [4]. Alkaline phosphatase was demonstrated by the Manheimer-Seligman method [14]. The von Kossa technique as described by Lillie [13] was used for demonstration of calcium phosphate in the cultures. Haematoxylin–eosin staining, van Gieson-Hansen staining and methylgreen pyronin staining (Leuchtenberger's technique [12]) were also used for special purposes.

Calcification and phosphatase reaction in Carrel flask cultures.—Cultures of heart fibroblasts in Carrel flasks, in the presence of organic phosphates, demonstrate calcification as a white ring in the peripheral part of the migration zone, as previously described [1]. This calcification usually begins after 4 or 5 days of incubation. By phosphatase staining of control cultures a ring-shaped red phosphatase staining appears in exactly the same locality as the circular calcification zone in the experimental cultures. The phosphatase staining was present about one day prior to calcification. Apart from the circular calcification zones in the periphery, calcium phosphate was found in the central parts of the cultures, and also along the cut edges appearing as border lines between the passages of the transferred coverslip culture. Phosphatase staining was usually absent from these parts of the cultures at the time of fixation. Because of this, and since Carrel flask cultures are inconvenient for histological work, phosphatase staining of Carrel flask cultures have not been performed on a large scale.

Spontaneous calcification in cover slide cultures of heart fibroblasts. Calcification in hanging drop cultures of heart fibroblasts cultivated in normal media appears in two different localizations. It occurs along the cut edges of cultures and also in the original fragment of embryo heart muscle, if this is preserved during cultivation. Calcification along the cut edge is not seen in all cultures, usually not in more than a few in a series of 25 cultures. It appears as thin, white, pearl-like deposits, or as a more continuous white precipitation along the cut edge. With the von Kossa technique, they are stained black (Fig. 1), and with oxalic acid, crystals of calcium oxalate can be formed. Therefore it must be assumed that the white precipitate is caused by calcium phosphate, and this assumption is in accordance with the findings of Hueper and Russel [11].

In sections of slightly calcified cultures (as well as in whole mounts), calcification is localized in the single spindle-shaped cells (Fig. 2). When calcification is more intense, the medium between the cells is also calcified.



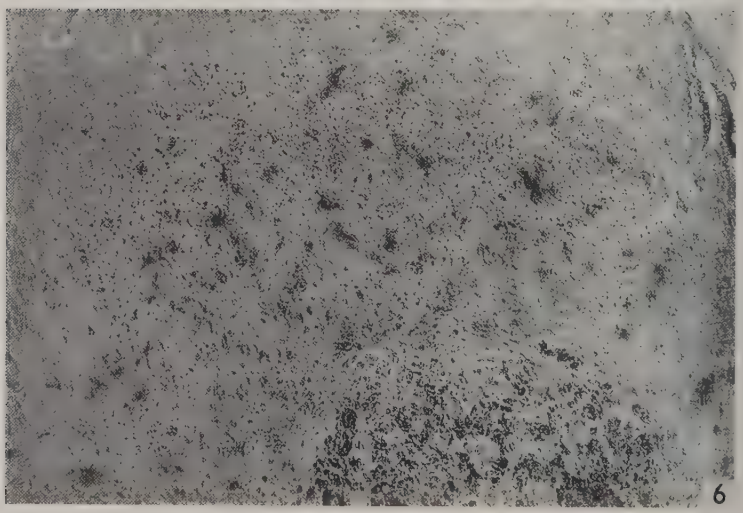


Fig. 5. Section of fibroblast culture, parts of explant of chicken embryo heart and of the marginal zone of the first passage. Von Kossa staining. In the central piece the calcification is only slight, localized in small round cells. In the periphery of the migration zone, along the cut edge, the calcification is intense. Towards the central piece the calcification is of decreasing intensity. Magnification × 145.

Fig. 6. Section of the same culture as Fig. 5. Manheimer-Seligman staining for alkaline phosphatase. Note that most of the phosphatase-stained cells are located in the uncalcified area. The most peripheral phosphatase-stained cells are located in the most central part of the calcified area inside the cut edge. Magnification × 145.

The calcification is seen to begin in the cells lying at or just inside the cut edge, spreading from there centrally towards the middle piece or to a more centrally placed cut edge. At the same time the calcification along the outer cut edge becomes more intense. Although the outer edge of the calcified area is always very sharp when seen macroscopically, a few cells outside the cut edge are often seen to be calcificated when observed in the microscope.

Calcification of the central piece, the heart fragment, starts in small roundish elements corresponding in shape to the embryonic cells of the heart (Fig. 5). Later on the calcification here also becomes more solid, so that the single cells can no longer be seen.

The relationship between calcification and alkaline phosphatase.—The areas which calcify in the cultures are exactly the same as those in which alkaline phosphatase activity was seen to develop in heart fibroblast cultures as previously described [10]. However, the two processes do not occur simultaneously. This had been observed in the experiments with calcification in Carrel flask cultures. Each single culture represents only the state at the time of fixation, but comparing the pictures of cultures of different ages, it is possible to observe the following steps in the relationship between alkaline phosphatase and calcification (Figs. 3–7).

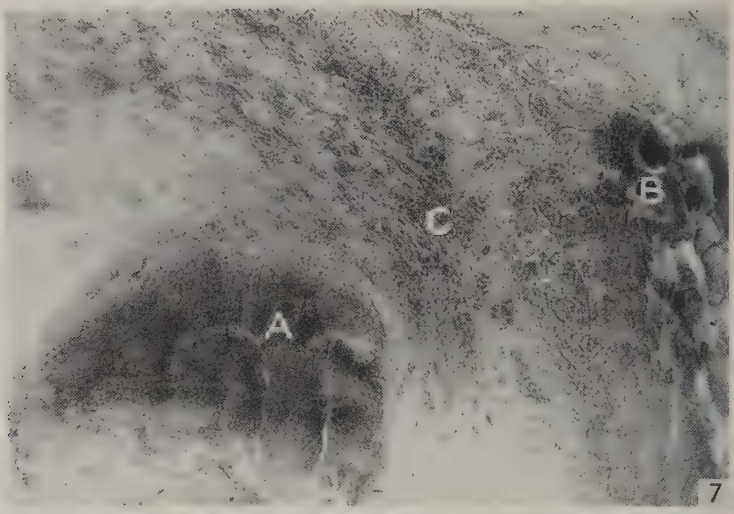
Stage I: Alkaline phosphatase is present, but there is no sign of calcification. The distribution of the alkaline phosphatase in the culture and alterations of the cell structure when alkaline phosphatase appears in a previously normal phosphatase-free fibroblast have been described elsewhere [10].

Stage II: Alkaline phosphatase and calcification are present at the same time. The calcification is only slight, and always localized in single cells. No calcification of the medium between the cells is seen at this time. In sections of heart fibroblast cultures this can be seen in the areas inside the cut edge and in the heart fragment, if this is preserved. This stage is obviously only present for a short time, since it is more common to find alkaline phosphatase without calcification (Stage I), or to find calcification without phosphatase (Stages III or IV).

Stage III: The stain for alkaline phosphatase is negative but calcification is present. The calcification is still localized in the single cells, but it is more pronounced than in Stage II.

Stage IV: Often the calcification develops further, so that the intercellular space also is calcified. When this happens the outlines of the single cells disappear and real calcareous masses are formed. As in Stage III the staining reaction for alkaline phosphatase is negative at this time.

Often it is possible in a culture to see all four stages at the same time, but



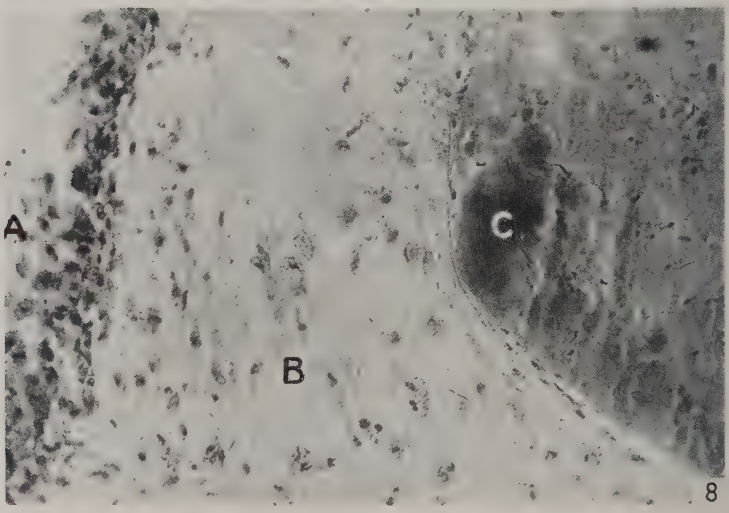


Fig. 7. Section of fibroblast culture, sixth passage. Manheimer-Seligman staining for alkaline phosphatase, counter staining with haematoxylin. Parts of the fourth and fifth passage. The calcified parts of the fourth and fifth passages are stained dark blue by haematoxylin (*A* and *B*). The red-stained, phosphatase-containing cells are located in the uncalcified area (*C*). Magnification × 285.

Fig. 8. Section of fibroblast culture, sixth passage. Staining with methyl-green pyronin. *A*, cells of the migration zone of the sixth passage. Here the nuclear staining is normal. *B*, cells of the fifth passage; these cells stain for alkaline phosphatase. Note the weak but visible nuclear staining in this area. *C*, calcified area of the fourth passage. Note the absence of nuclear staining in the peripheral part of the calcified area and the weak nuclear staining in the more central part.

in different parts of the culture. The most peripheral part of an area inside a cut edge is in Stage III or IV, the most central part in Stage I and the intermediate part in Stage II. When cultures of heart fibroblasts grown on perforated cellophane, as previously described, are stained by the Manheimer-Seligman method, and then by the von Kossa method, the same sequence of phosphatase staining and calcification is seen. After only one day of cultivation no phosphatase staining has yet appeared. After 2–3 days phosphatase reaction appears in some of the migrating cells and in some parts of the explant itself. As a rule calcification is not yet present at this time. About one day later calcification begins to appear localized in single cells, some of which still stain for alkaline phosphatase, while others are negative. After 4–5 days this condition is still found in some of the cells, but in many areas a heavy calcification has developed, which stretches outside the limits of the cells. In these areas no phosphatase is found.

Stage III could possibly be the picture of Stage II from which the alkaline phosphatase had disappeared. However, as Stage IV appears in which the calcification is found outside the boundaries of the cells, i.e. in places where alkaline phosphatase activity is never seen in the cultures, it seems permissible to conclude that the process of calcification is a diphasic one. The alkaline phosphatase is active only in the first phase, in the second phase the calcification proceeds at a time and a place where alkaline phosphatase activity is not present, possibly by growth of crystals.

The relationship between phosphatase staining, calcification and nuclear staining is of special interest. As staining by haematoxylin is of no value in calcified areas where there is a strong background staining with haematoxylin, staining with methyl-green was used.

As a rule staining of the nucleus was observed in the phosphatase-stained cells, although weaker than in the normal phosphatase free fibroblasts (Stage I). Also in slightly calcified cells a weak nuclear staining could sometimes be seen, but as a rule no nuclear staining was seen in calcified cells (Stage II). The gradual disappearance of the nuclear staining in normal cells, through the stage of phosphatase-stained cells, to the stage showing cells in calcified areas is clearly seen in several preparations (Fig. 8). Calcification in heart fibroblast cultures is apparently a calcification of necrotic cells, such as was previously described by von Möllendorff [15].

Robison's "first and second factors".—When phosphatase staining first appears (before calcification begins), we have –using the terms of Robison—the "first factor", the alkaline phosphatase; but the "second factor" or "local

factor" is absent. When calcification sets in some time later both factors must be present.

Is it possible to gain, from the histological facts, any information about the second or local factor? The changes in the nuclear staining could be of interest in this connection. Robison assumed that alkaline phosphatase effected its action by setting free PO_4^{---} from organic phosphate compounds, and that the effect of the local factor consisted in making the tissue capable of binding the calcium phosphate. The absence of calcification in e.g. kidney tissue *in vitro* in the presence of organic phosphate and calcium ions as seen in the experiments of Rosenheim and Robison [19] was thought to be due to lack of the local factor. Experiments of this kind, of which many have been performed, are in reality a phosphatase staining by the method of Gomori [18] done as a vital staining. If the assumption of Robison and co-workers were correct, one should expect it to be possible to determine the organic part of the split molecule, i.e. the beta naphtole residue in the Manheimer-Seligman method [14]. However, this is not possible, as will be seen from the following experiment:

A rat was killed with chloroform and the kidneys were immediately removed and cut in thin slides, some of which were fixed in 80 per cent alcohol, while others were placed in sterile Ringer's solution. The alcohol-fixed slides were brought down to water. On all slides, fixed and unfixed, a Manheimer-Seligman phosphatase staining was then performed at pH 7.0, 7.5, 8.0, 8.5, 9.0 and 9.3 using Ringer's solution instead of water.

The fixed slides showed normal phosphatase staining. The kidney tissue was stained red in the cortex, the staining being somewhat weaker at pH 7.0 than at a higher pH. In the unfixed slides no staining at all could be seen. A similar experiment was made with unfixed bones (femora and tibiae) from chicken embryos 15–18 days old. In this material there was a normal-looking strong phosphatase staining of the periostium and of the bone mantle, i.e. the parts in which calcification is going on; but there was no phosphatase staining of the uncalcified hypertrophied cartilage, which is stained in fixed preparations.

The negative phosphatase reaction could apparently not be due to a lack of contact between the phosphatase and its substrate. The low molecular weights of the naphthol phosphate and of the diazo compound used for coupling would make it difficult to explain why diffusion should be possible in the cells of the periostium and not in kidney cells and cartilage cells. It seems appropriate to assume that phosphatase present in unfixed kidney slices neither attacks the naphthol phosphate nor the hexose phosphate or the glycero phos-

phate used by Robison and co-workers. We must assume that the action of the alkaline phosphatase inside a living cell differs in some way or other from the action observed in test tube experiments. The enzyme action which can explain the function of the alkaline phosphatase in calcification, appears when the cells are dying either as the ultimate result of cell degeneration (as in the tissue cultures here described) or as a result of fixation (as obtained in the normal Gomori phosphatase staining procedure).

DISCUSSION

Von Möllendorff [15] has previously described calcification in the middle piece and along the cut edges in tissue cultures. Fischer, Laser and Meyer [7], Demuth [3] and Hueper and Russel [11] have described the ring-shaped calcifications which appear in Carrel flask cultures. Fischer *et al.* and Demuth supposed that the precipitations were calcium lactate, but Hueper and Russel showed that they were composed of calcium phosphate and calcium carbonate.

Von Möllendorff investigated the middle piece of fibroblast cultures. The descriptions of calcification and the pictures shown in his paper indicate that the localization of the calcifications in his cultures are the same as described here. He found calcification to occur in the original explants in areas with severe alterations of the cells, such as formation of pigment (granules?), disintegration (*Zerfall*) of the nucleus and of the cytoplasm. With respect to the calcification in the migration zone, i.e. along the cut edges of the culture, he has especially observed the advanced degree of calcification which includes calcification of the medium between the cells. He has not observed the beginning of the calcification and its localization in the single cell. He assumes that a differentiation into osteoid tissue has taken place and discusses therefore the conditions for this "differentiation". Von Möllendorff has not studied the phosphatase reactions, hence it has not been possible for him to discuss the connection between alkaline phosphatase and calcification.

We know now that alkaline phosphatase appears during degeneration of fibroblasts [10], so there is no reason to consider calcification in fibroblast cultures as a phenomenon of differentiation into bone tissue.

The calcification described by Hanks [9] is in some way quite different from the calcification described here. Hanks found that calcium phosphate would precipitate in a fluid composed of equal parts of serum and embryonal extract. This took place when the solubility product of Ca^{++} and PO_4^{---} was exceeded as a consequence of the formation of free phosphate by the

action of the alkaline phosphatase present in the embryo extract. The precipitation took place even if no cells were grown in the medium, but it is of interest to notice that when a culture was present calcification began close to the explant. Hanks writes: "Growing cell colonies obviously play a role in phosphate liberation and calcium phosphate deposition near the central explant." Staining for alkaline phosphatase has not been performed by Hanks, but one may assume that alkaline phosphatase activity of the cells as described here have caused the precipitations localized in the cells.

The observations presented in this paper lead to the following conclusions:

(1) The action of alkaline phosphatase inside a living cell will not cause calcification.

(2) The *initial* calcification takes place when alkaline phosphatase is liberated from a severely damaged, dying or dead cell containing phosphatase.

(3) The phosphatase then disappears but the calcification proceeds and spreads outside the limits of the cell (into the medium).

The results of the action of the alkaline phosphatase inside a living cell differ from the results obtained with test tube experiments with liberated phosphatase. The nature of this difference is however still unknown. The alkaline phosphatase might react in the enzymatic processes inside the living cell, so that its action on a substrate other than the natural may only be slight. The PO_4^{---} which is split off by the enzyme under natural conditions might immediately be taken over by other enzymes and built into new compounds, so that no accumulation of PO_4^{---} takes place. It must be assumed that a correlation of the different enzyme systems in a living cell is one of the fundamental processes of life. If we assume that there is such a cellular function, it is not difficult to explain why calcification does not take place when alkaline phosphatase is present in living cells; and it is unnecessary to operate with "lack of a second factor".

If we assume such a difference between the action of the phosphatase inside a living cell and the action after the phosphatase has been liberated when the cell is dying, it is not difficult to explain why there is a close relationship between calcification and histological signs of cell death. The correlated functions of the various enzymes in the cell are interrupted; the alkaline phosphatase reacts, as we know, from test tube experiments without living cells; phosphate ions are split off from organic compounds. There is an accumulation of PO_4^{---} and calcium phosphate is precipitated or crystals of apatite are formed [2], if Ca^{++} is present in sufficient amounts. When Robison's phosphatase theory is supplemented by this hypothesis the unsolved

problem of the hypothetical second or local factor disappears. The question is whether the alkaline phosphatase acts as free phosphatase or not. The present hypothesis explains why calcification experiments such as those performed by Rosenheim and Robison with living kidney tissue are not successful, while calcification in the kidney tubules promptly appears with the Gomori staining for alkaline phosphatase when the kidney is killed in such a way that the alkaline phosphatase is not destroyed (alcohol or acetone fixation) and the chemical composition of the tissue is not severely altered (freeze-drying). The pathological calcification in the areas of the kidney tubules containing alkaline phosphatase, as seen in mercuric chloride poisoning, are explained in the same way.

The experiments indicate that the reaction for alkaline phosphatase was positive when calcification began, namely in that phase of the calcification process in which the calcification is still localized in the single cells. Later, when the calcification was more extensive and included the intercellular space, no phosphatase staining could be seen in that part of the fixed preparations. Therefore the alkaline phosphatase is active only in the first step of calcification, which when first started proceeds without action of any local phosphatase. In this step of calcification in which the initial calcification caused by the phosphatase seems to act as a trigger mechanism, the explanation may be found of the many experiments of calcification *in vitro* using unphysiologically, supersaturated solutions of inorganic salts, especially when all enzymes are destroyed [21]. The experiments described by Hanks, which demonstrate a rise of the phosphate ion concentration of serum, could perhaps find a place in a calcification scheme as part of the humoral conditions for calcification.

It is understood that this revision of Robison's phosphatase theory has not solved all the problems of calcification; but the hypothesis is useful in experimental investigations. It is in accordance with the histological facts concerning the relationship between alkaline phosphatase, nuclear stains and calcification in tissue cultures.

SUMMARY

The relationship between alkaline phosphatase, cellular alterations (degeneration, cell death) was studied in cultures of heart fibroblasts. Histologically the following stages could be observed:

Stage I: Alkaline phosphatase present in cells showing signs of degeneration; nuclear staining present, but weaker than in normal cells; no calcification.

Stage II: Alkaline phosphatase present in cells without nuclear staining by methyl-green. Calcification present, localized in single cells.

Stage III: Negative stain for alkaline phosphatase; negative nuclear staining by methyl-green; calcification present, localized in single cells.

Stage IV: As Stage III, but calcification is found outside the cell boundaries.

It was previously known that the method of Gomori is unsuitable as a vital stain for alkaline phosphatase in areas which do not calcify. Results presented here indicate that this is also true in using the method of Manheimer and Seligman. Alkaline phosphatase reacts in different ways, depending on whether it is inside a living or a dead cell.

It is concluded that the process of calcification is of diphasic nature. The initial calcification takes place when the alkaline phosphatase is liberated from dying cells containing phosphatase. Thus, an explanation of the initial stage of calcification is proposed, which avoids the use of a "local or second factor" found in Robison's theory. The calcification caused by the phosphatase serves as a trigger mechanism in the second phase of the calcification, which takes place without action of alkaline phosphatase.

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ULTRASTRUCTURE DU CORPS PARANUCLÉAIRE, DES MITOCHONDRIES ET DE LA MEMBRANE NUCLÉAIRE DES GAMÈTES D'*ALLOMYCES MACROGYNUS*

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Contrairement à l'opinion de Hatch [11], qui considérait le corps paranucléaire (nuclear cap) des gamètes d'*Allomyces* comme un amas de mitochondries vésiculisées, nous avons récemment pu montrer, par voie cytochimique, que cette formation cytoplasmique représente une accumulation transitoire d'acide ribonucléique associé à des protéines sulfhydrilées [27], totalement indépendante des chondriosomes.

Cependant, nos observations sur la présence de mitochondries dans le cytoplasme entourant le corps paranucléaire, tant par microscopie optique normale, après coloration vitale au vert Janus, que par microscopie à contraste de phase, restaient incertaines par suite des dimensions réduites de ces organites.

Il appartenait donc à la méthode moderne de l'observation au microscope électronique, sur coupes ultra-minces

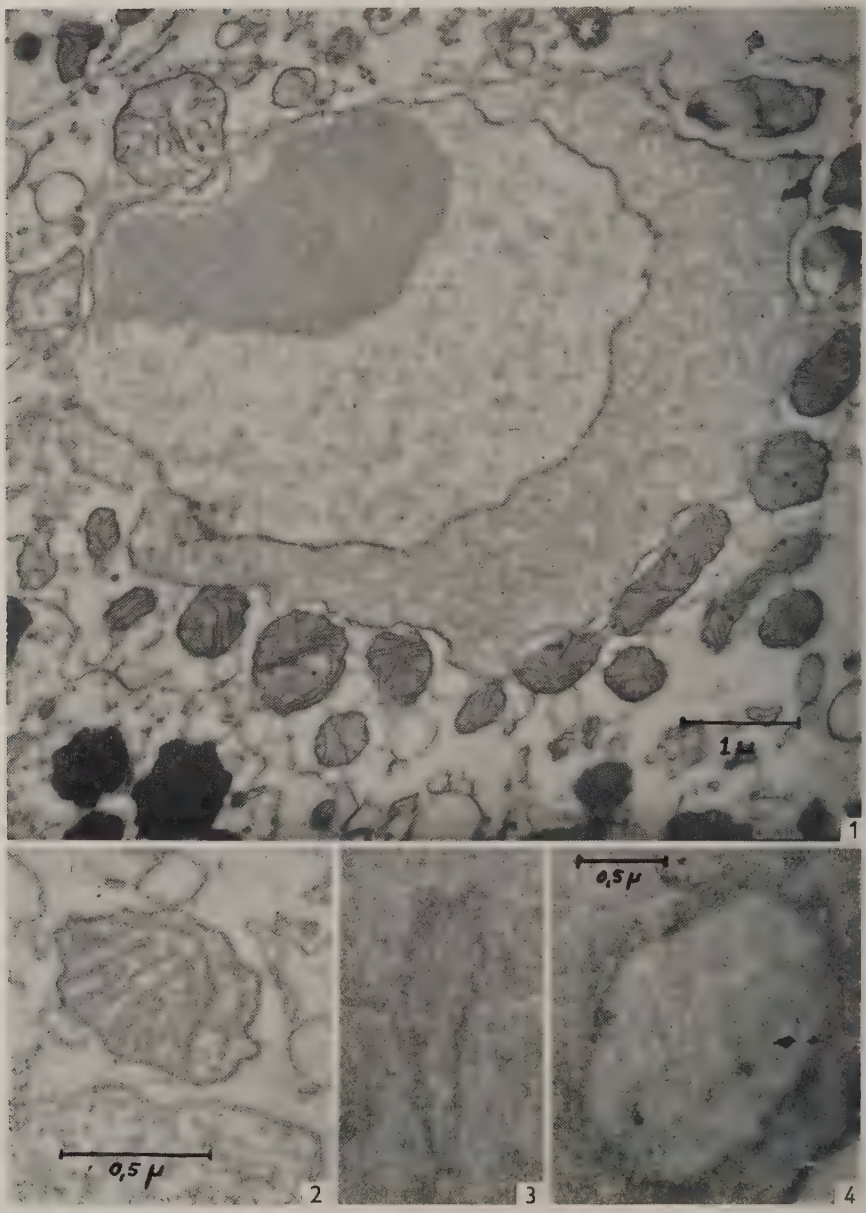
— de confirmer l'indépendance du corps paranucléaire basophile et des chondriosomes;

— de préciser si le corps paranucléaire — qui concentre la totalité de la basophilie du cytoplasme gamétique à l'exception des granulations métachromatiques [27, 28, 29] — présente une structure fine permettant de le comparer à un ergastoplasme typique [3];

— de nous renseigner sur l'ultrastructure des chondriosomes et de la membrane nucléaire.

MÉTHODES

Les gamètes d'*Allomyces macrogynus* Emers. et Wils. [8] (= *A. javanicus* Kniep var. *macrogynus* Em.) proviennent de colonies gamétophytiques cultivées 10 j. sur milieu amidon-extrait de levure et immergées 2 3 h. dans une solution saline neutre (solution DS de Machlis [15]). La suspension de gamètes obtenue est fixée 5 min. dans OsO_4 à 0,2 % en tampon acétate-véronal à pH 7,2. Une centrifugation précède


1 μ
1
0,5 μ
2
3
0,5 μ
4

la fixation définitive dans OsO_4 à 2 % (pH 7,2) pendant 4 h. Après nouvelle centrifugation, le culot de gamètes est lavé dans le tampon acétate-véronal, avant d'être incorporé dans 0,1 ml d'agar à 1,5 % (tamponné aussi à pH 7,2) afin de former de petits blocs facilitant les manipulations subséquentes de deshydratation par la série des alcools et de transfert dans le butyl-métacrylate (selon Kellenberger et Ryter [12]). L'inclusion est effectuée dans le butyl-métacrylate avec 5 % de méthyl-métacrylate. La polymérisation est réalisée en présence de 0,5 % de peroxyde de benzoyle, à 55°C pendant 24 h., dans les capsules de gélatine habituelles.

Les coupes sont confectionnées à l'aide d'un ultramicrotome décrit ailleurs [13]. Nous avons employé des lames de rasoir (PAL) aiguisées selon la méthode de Sjöstrand [26].

Microscope électronique RCA EMU-2D.

RÉSULTATS

L'examen des coupes obtenues permet de reconnaître un premier ensemble structural comprenant le corps paranucléaire emboîtant le noyau et son nucléole (Fig. 1). Ces trois formations ne présentent pas une structure définie; elles diffèrent essentiellement par la densité de leur matière constitutive. Celle-ci est la plus dense et la plus homogène dans le nucléole. A cet égard, le corps paranucléaire présente des caractères intermédiaires entre le nucléole et le noyau.

La membrane nucléaire, séparant noyau et corps paranucléaire, révèle, en coupe transversale (Fig. 1), l'aspect d'une structure double parsemée de pores (grande dimension, environ 950 Å), telle qu'elle a déjà été décrite chez de nombreux organismes [30]. Il paraît en être de même pour la membrane séparant le corps paranucléaire du reste du cytoplasme.

Nous avons eu la bonne fortune d'obtenir des coupes tangentielles de la membrane nucléaire dans la région du corps paranucléaire (Fig. 3). Nous y observons une structure alvéolaire régulière. Fait notoire, les contours des alvéoles sont tous fermés. Une structure homogène, plus dense et plus fine que le "paranucléoplasme", entoure cette zone alvéolaire. Dans une coupe oblique (Fig. 4), nous retrouvons des alvéoles à contours fermés, sauf deux dont le pourtour est ouvert en direction du noyau.

Les chondriosomes sont concentrés sur le pourtour du corps paranu-

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Fig. 1. Vue d'ensemble d'une coupe de gamète d'*Allomyces macrogynus* Emers. et Wils.
Fig. 2. Détail d'un chondriosome, à fort grossissement. Sur le bas de la figure, portion du corps paranucléaire avec sa membrane.
Fig. 3. Coupe tangentielle dans la membrane nucléaire au niveau de la structure alvéolaire et corps paranucléaire.
Fig. 4. Coupe oblique dans la membrane nucléaire avec nucléoplasme au centre et matière paranucléaire sur le pourtour.

cléaire (Fig. 1). Ils sont du type classique tel qu'il a été décrit dans les cellules somatiques des Vertébrés [17, 21, 25, 26] et de quelques Invertébrés [7, 9], avec une membrane périphérique et des lamelles transversales internes (cristae mitochondriales selon Palade [16], Fig. 2). Les petits granules noirs, également observés de manière constante dans les mitochondries animales [17, 21, 25, 26], se retrouvent aussi dans les chondriosomes d'*Allomyces*.

Nous avons trouvé, sur certaines de nos coupes, un organite placé en face du nucléole (Fig. 1) et dont la structure l'apparente aux chondriosomes. Cependant, de par sa moindre densité et sa position dans la cellule, cet organite pourrait être distinct des chondriosomes et appartenir à l'appareil locomoteur, bien développé dans les cellules mobiles d'*Allomyces*.

Quant à la structure fondamentale du cytoplasme périphérique, elle se prête mal à une description et nous pensons que sa fixation est encore inadéquate. Néanmoins, on peut déjà affirmer qu'il ne s'y trouve aucune structure ressemblant à l'ergastoplasme typique tel qu'il est décrit dans les cellules animales (complexe de membranes et grains de Palade [3, 18]). Seules les membranes et les petites formations ballonnées visibles dans ce cytoplasme périphérique pourraient avoir quelque parenté avec la composante membraneuse de l'ergastoplasme (« endoplasmic reticulum » [20]). La composante granulaire en est par contre absente, ce qui se traduit d'ailleurs par l'absence de basophilie dans cette zone cytoplasmique [27].

Les grosses granulations osmiophiles disséminées dans le cytoplasme périphérique peuvent correspondre aux granules lipidiques, riches en phosphatides et en caroténoïdes (gamètes mâles).

DISCUSSION

L'étude micromorphologique des gamètes d'*Allomyces macrogynus* a donc apporté la confirmation des observations cytochimiques (Turian [27]), à savoir que le corps paranucléaire basophile est bien distinct des chondriosomes. Rappelons que ces derniers se présentent sous forme de longs chondriocontes dans les hyphes végétatifs d'*Allomyces* [11, 22].

La similitude ultrastructurale des chondriosomes d'*Allomyces* avec les mitochondries animales du type classique, à lamellation interne (celles des Protozoaires tels que *Paramecium* [24]; *Tetrahymena* et *Tokophrya* [23] sont du type à « microvillosités » internes), souligne les affinités animales des Champignons. La complexité structurale des chondriosomes d'*Allomyces* n'est, en effet, pas comparable à la différenciation encore confuse des chondriosomes végétaux, du *Fucus vesiculosus* par exemple [14].

Nos observations concernant la membrane nucléaire rappellent celles faites par Bairati et Lehmann [1] ainsi que Harris et James [10] sur la membrane nucléaire de l'Amibe et celles d'autres auteurs [2, 4, 5] sur divers types cellulaires. La zone plus foncée autour du groupe alvéolaire et le fait que les alvéoles ont un pourtour fermé sur la Fig. 3 nous conduisent à admettre l'existence d'au moins une membrane continue, en position externe par rapport à la couche alvéolaire. Nous ne pouvons pas exclure la possibilité que cette dernière ne soit pas recouverte à l'intérieur par une deuxième membrane continue. Quant au rapport entre les alvéoles, visibles sur les coupes tangentielles et obliques, et les pores, apparents sur section transversale, il est encore obscur. Il faudra cependant tenir compte à l'avenir de l'existence de tels pores dans la membrane nucléaire pour mieux comprendre le problème de la genèse du corps paranucléaire lors de la différenciation des gamètes dans les gamétanges d'*Allomyces*. Une participation, du moins directe, des chondriosomes à cette formation [11] doit, en effet, être désormais exclue. Le contact direct des substances nucléaires et paranucléaires au travers des pores de la membrane plaide en faveur d'échanges nucléo-paranucléaires et l'hypothèse de Caspersson [6], selon laquelle des substances basophiles en provenance du nucléole seraient libérées dans le cytoplasme, trouve peut-être chez *Allomyces* une illustration particulièrement favorable.

La présence d'un corps paranucléaire ribonucléique, concentrant à lui seul la presque totalité de la basophilie cytoplasmique, n'est l'apanage que des cellules hautement différenciées que constituent les cellules mobiles — gamètes, planozygotes et zoospores — chez *Allomyces*. Par contre, dans les zygotes en germination, sièges d'un phénomène de dédifférenciation (rejuvénescence) initié par la « résorption » du corps paranucléaire, la basophilie gagne progressivement l'ensemble du cytoplasme [29].

Nous avons montré plus haut que le corps paranucléaire ne contient certainement point de constituant membraneux (« endoplasmic reticulum »). Selon certains indices, l'acide ribonucléique s'y présenterait sous la forme granulaire. D'autre part, le cytoplasme périphérique des gamètes est exempt de grains de Palade, alors qu'on peut y entrevoir l'existence de la composante membraneuse. Ces faits nous conduisent dorénavant à l'intéressant problème de savoir si l'extension de la basophilie à l'ensemble du cytoplasme, conséquence de la « résorption » du corps paranucléaire ribonucléique, se traduit ou non, au niveau submicroscopique, par l'apparition d'un ergastoplasme complet (« rough surfaced variety of endoplasmic reticulum » [19]) dans la jeune plantule d'*Allomyces*.

SUMMARY

1. Ultrathin sections through gametes of *Allomyces macrogynus*, when observed in the electron microscope, show sharp separation between mitochondria and nuclear cap.

2. No typical ergastoplasm is found neither in the basophilic (RNA) nuclear cap nor in the peripheral cytoplasm of the gametes.

3. Mitochondria of gametes of *Allomyces* exhibit the classical structure described for animal mitochondria.

4. The nuclear membrane is a double structure; pores in this structure are visible in transverse sections. A regular alveolar structure is seen on tangential and oblique sections; this alveolar structure is limited externally by a continuous membrane.

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ULTRASTRUCTURE OF THE SYNAPSE IN THE GIANT AXONS OF THE EARTHWORM

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The function of the giant axons in the earthworm is that of mediating rapid synchronous distribution of impulses to an extensive musculature. These axons are located in the ventral nerve cord and are divided at regular intervals along their length into segments by septa [39], which are oblique partitions thrust across the giant axons. The septa, which originate in the sheath of the fibers, are the sites of synaptic relations between adjacent segments. They provide a broad area of contact between the two axoplasms and represent synapses of unusual proportions [6]. The synaptic nature of the septa is further supported by the fact that degeneration of the axons after sectioning does not cross these boundaries [39]. A study of the ultrastructure of these synapses has not been made so far. In view of the fact that extensive neurophysiological information is available about the giant axons, it was thought that an investigation of their structure with the electron microscope should prove of interest in attempts to correlate morphology and function.

MATERIALS AND METHODS

The ventral nerve cord of *Lumbricus terrestris* was used throughout this investigation. The worm was anesthetized in 0.2 per cent chloretone solution in isotonic saline [40] and pieces of the cord were dissected out and immediately dropped into the fixative. The fixatives used were 10 per cent formalin, and 2 per cent osmium tetroxide solution buffered at pH 7.3 with acetate veronal buffer [24]. After fixation for twelve hours in osmic acid or twenty-four hours in formalin, the tissue was dehydrated and then placed in a mixture of equal parts of absolute alcohol and methacrylate monomere for one hour. The methacrylate monomere is a mixture of equal parts of ethyl methacrylate and *n*-butyl methacrylate with the inhibitor removed (instructions from the manufacturer).

Infiltration was carried out with pure monomere for twelve hours under refrigera-

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tion (5°C). The tissue was then embedded in fresh monomere mixture, placed in gelatine capsules and the plastic was polymerized by ultra-violet light for six hours [18]. Sections were cut at 0.1–0.3 μ with a glass knife [16]. After mounting, the sections were freed of the embedding material with toluene. This last procedure was consistently carried out after preliminary observations showed that small differences in density in the structures involved were considerably obscured by the embedding medium. The electron micrographs were taken with an RCA model EMU electron microscope at original magnifications ranging between 2000 and 24,000 diameters and enlarged further in printing as indicated in the explanation of the figures.

OBSERVATIONS

The axons of the earthworm nerve cord contain two distinct structural elements which have a constant distribution in all the specimens studied. The first of these elements is a mass of axoplasmic filaments with nodose outline and a diameter of 165–200 Å. The second of these elements is a large number of dense neurofibrils with smooth contours, a uniform diameter of about 0.1 μ and a complex internal structure. The giant axons (Fig. 1) contain almost exclusively interlaced axoplasmic filaments and no neurofibrils except near the synapse where neurofibrils appear on the preneuron side of the septum. The smaller axons of the neuropile (Figs. 2 and 3), however, contain both axoplasmic filaments and neurofibrils, which are found along the entire length of the processes. The neurofibrils have varying lengths in our figures. It may be that this appearance is due to sectioning of contorted fibrils of indefinite length, however, this is difficult to determine.

The neurofibrils are seen as separate entities or in groups. When they cross and overlap the area of crossing, or overlap, has a higher electron density, a fact which indicates that the neurofibrils do not anastomose, but retain their individuality in the group.

Under higher magnification each axoplasmic filament (Fig. 4) appears as a "string of beads" formed by dense particles, 165–200 Å in diameter, which are joined by material of very low density (Fig. 4a). The particles are more or less regularly spaced. In some filaments, however (Fig. 4b), the particles are packed close together giving to the filament a smooth outline. There are also areas where the particles, although retaining a linear arrangement, are widely spaced and appear disconnected (Fig. 4c). If there is some material joining them it has a very low density to the electron beam.

The neurofibrils under higher magnification present a complex internal structure (Figs. 5 and 6). They appear to consist of a smooth sheath of medium density and of a dense strand of material forming a spiral in their

interior (Fig. 6). Very often the end of a neurofibril continues as a parallel bundle of axoplasmic filaments (Fig. 5) or frays out as an entangled mass of filaments (Fig. 6). These facts suggest that axoplasmic filaments are a major component of the neurofibrils. This is further supported by the observation of a structure which appears to be an intermediate stage in the formation of a neurofibril from axoplasmic filaments (Fig. 7). Distinctly nodose filaments fray out from the left end of the structure. On the right side the filaments are seen to form a parallel bundle, then some of these spiral for one turn and the entire bundle frays out again into nodose filaments. Occasionally, in this material, two neurofibrils may be twisted around each other in a rope-like arrangement (Fig. 7, arrows).

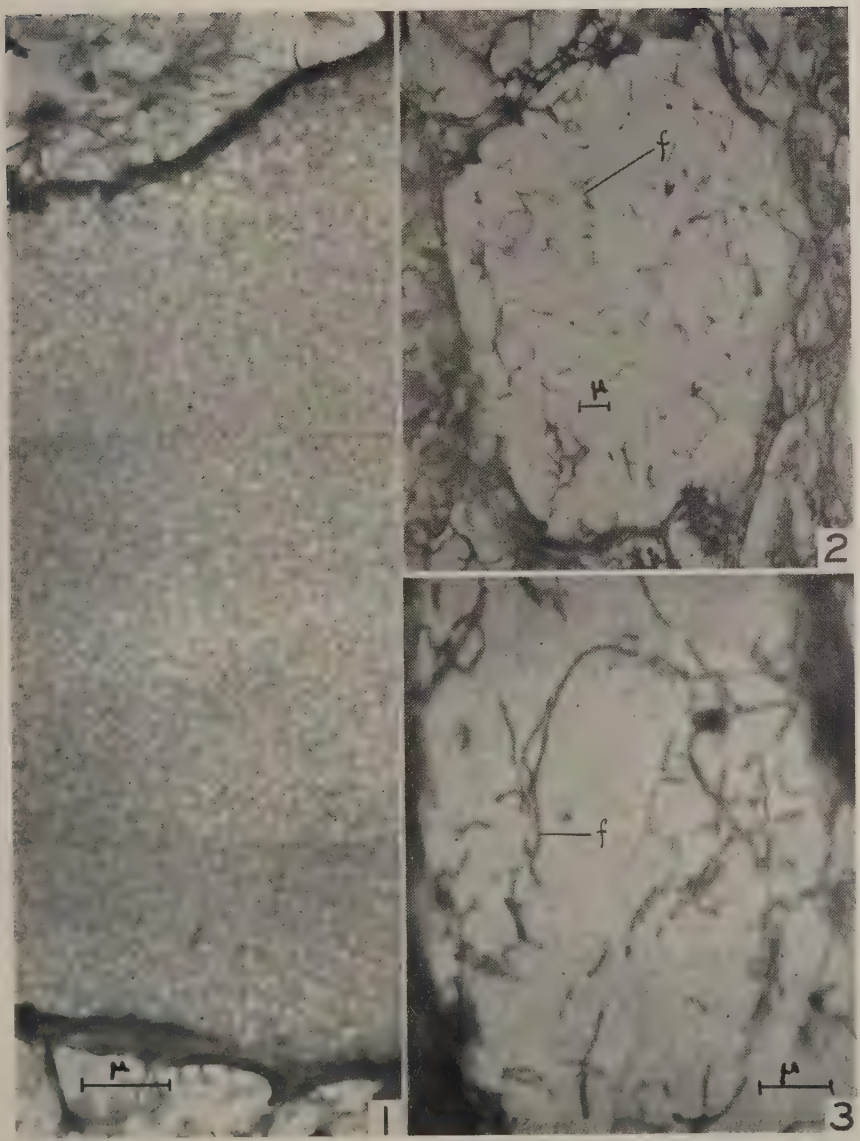
Fig. 8 represents one of the lateral giant axons at the level of a synapse. It is a cross-section through the trough-like oblique septum, the profile of which extends from the middle left to the upper right side of the figure. The sheath separates into concentric rings from which thin, ribbon-like lamellae peel off. These occupy the entire space between the axoplasm and the surrounding connective tissue of the nerve cord. Membranous lamellae also occupy the entire space between the axoplasms of the lateral and median axons. The line of contact of the circular sheaths of these two axons is indicated by a dip of the surrounding connective tissue (Fig. 8). The removal of the embedding medium has disrupted somehow the concentric arrangement of the sheath lamellae. Crevices appear between neighboring lamellae, also dense bands wherever the osmiophilic lamellae have remained closely associated. The low density of the individual membranous lamellae, however, necessitated the removal of the plastic.

The septum (Figs. 8, 9 and 10) also consists of a large number of lamellae which in this case separate the pre- and postsynaptic axoplasms. It is identical in structure to the sheath from which it originates. The lamellae are best seen in the areas where they are spread apart. In several places on the septum, however, the lamellae remain associated and appear as dense osmiophilic bands. No structural elements are observed between the membranous lamellae. There is no evidence in this material for the existence of an axolemma, i.e. a membrane differing in its electron optical and structural characteristics from the lamellae of the sheath and septum. However one of these lamellae (Fig. 8, am_1) invests the axon and is seen as a continuous structure around the axoplasm. Starting as the innermost lamella of the sheath, it can be followed up into the right side of the figure where it curves around and comes down as the outermost lamella of the septum on its lower surface. Because of this position it is termed the axon membrane. Axoplasmic fila-

ments are found on both sides of this synapse (Fig. 9). Neurofibrils, however, are present only in the lower, presynaptic member. The postsynaptic axoplasm contains in addition to axon filaments also small particles and dense masses of substance. Fig. 10 shows areas of the septum where the lamellae are closely associated into a dense band alternating with areas where they are spread apart as a result, it is believed, of the experimental procedure. It also indicates that numerous membranous lamellae may take part in the structure of the macrosynapse. In formalin-fixed material (Fig. 11) excessive coagulation of the axoplasm into larger fibers obscures the finer relationships. Considerable distortion of the tissue is observed. There are areas, however, (Fig. 11, *sy*) where the dense septum breaks up into a parallel array of filaments representing cross sections of thin lamellae.

In the smaller axons of the neuropile the two members of a monosynaptic junction are also separated by an oblique septum (Fig. 12, *sy*). This partition is considerably more delicate here than in the giant axons. It consists of two membranes running parallel at a constant distance from each other. A thin membrane (Fig. 12, am_1, am_2) invests each axon and is structurally identical to the membranes at the synapse and to the lamellae of the sheath and septum of the giant axons. The thickness of each axon membrane at the synapse (Fig. 13) is about 85 Å. In this preparation the membranes were oriented obliquely to the direction of the electron beam thus showing enhanced contrast along their cut margins in the upper surface of the section. Some dense material is seen in the area where they overlap. It cannot be determined, however, if it lies between the membranes or on the inner surface of am_1. Both members of this synapse contain fine nodose filaments and distinct neurofibrils which reach to the synaptic membranes but do not penetrate them. In the other axons seen in Fig. 12 the axoplasmic filaments are more or less numerous and neurofibrils are preferentially oriented parallel to the long axis of the processes. Dense granules and larger masses of substance are seen between axons. The axon membranes are very thin. Wherever small axons are packed together in longitudinal bundles their boundaries are therefore difficult to resolve.

Fig. 1. Tangential section of a giant axon showing interlaced axoplasmic filaments. Note complete lack of neurofibrils. Osmic acid fixation. × 13,000.
Fig. 2. Cross section of a large axon in the neuropile showing axoplasmic filaments, barely visible at this magnification, and neurofibrils (*f*). Osmic acid fixation. × 4000.
Fig. 3. Oblique section of an axon in the neuropile showing distinct axoplasmic filaments and neurofibrils (*f*). The sheath is seen on the left edge and in the upper right corner of the figure. Osmic acid fixation. × 10,000.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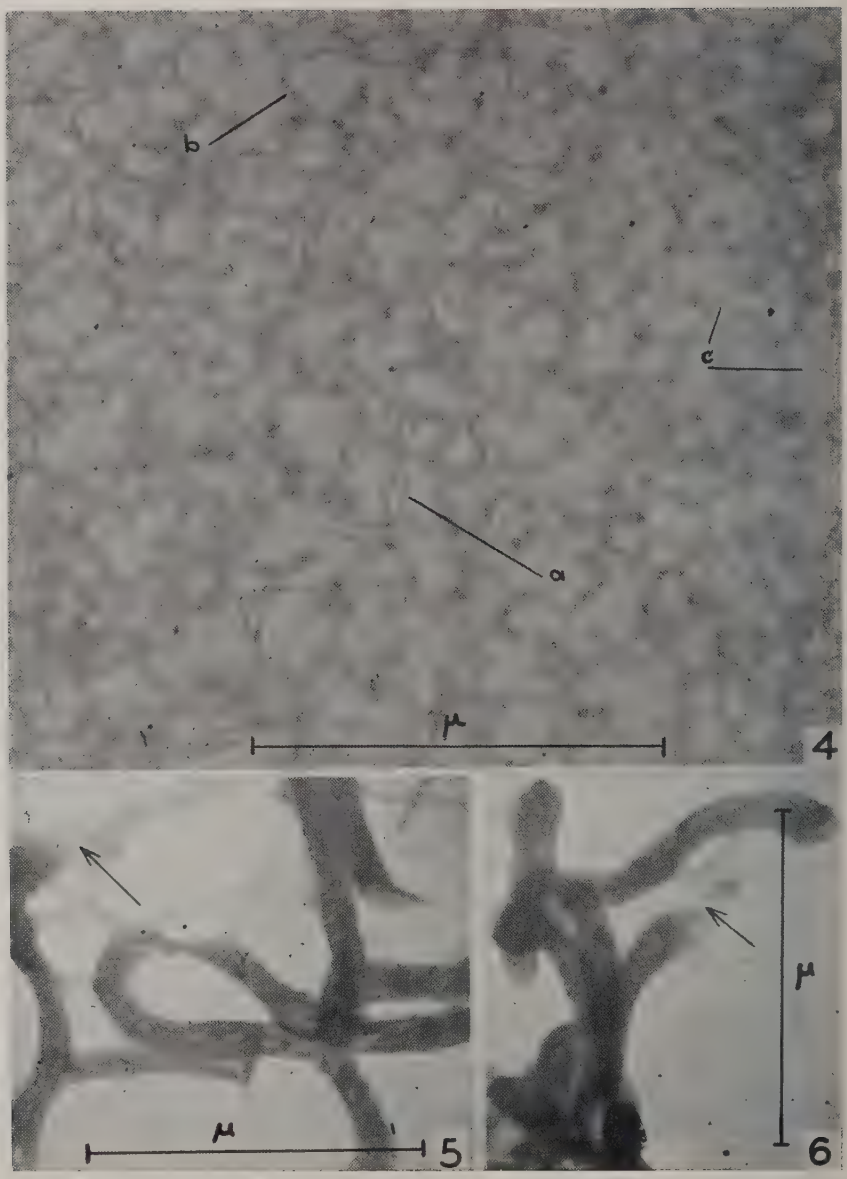
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DISCUSSION

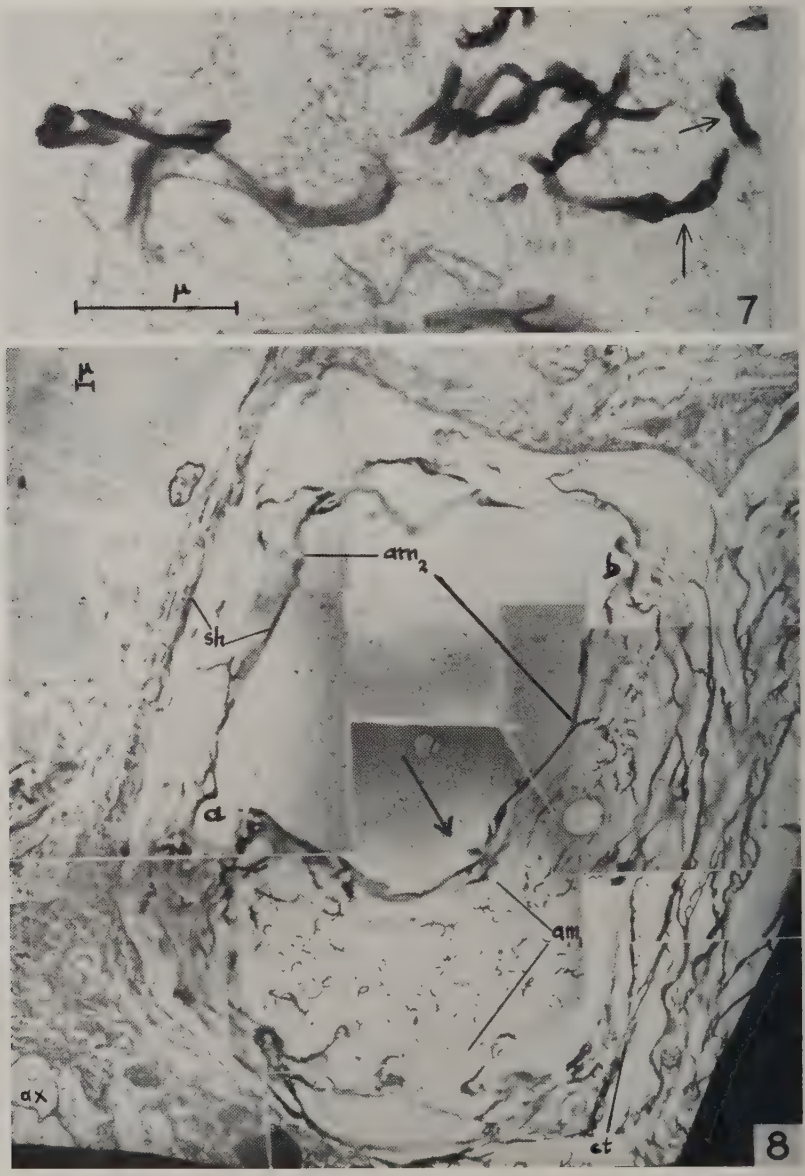
It is generally agreed that the axons of invertebrates [34] as well as of vertebrates [4, 12, 13, 20, 33] contain thin filaments of indefinite length and constant diameter. Depending upon the technique of preparation these may have a nodose or smooth appearance [9, 12, 20]. In a recent study of the earthworm neuropile with the electron microscope [8] axoplasmic filaments were not recognized with certainty. In the electron micrographs presented in this paper, however, they constitute the major part of the giant axons and are present in all other axons of the neuropile. Their appearance further suggests that they are formed by a linear aggregation of dense particles which also exist as individual units. These observations support the belief [19] that the chief constituent of axon filaments is a fibrous protein which has been shown to undergo reversible transformations into particles of lower molecular weight.

Earlier investigations with the light microscope [1, 38] have described extensively branched neurofibrils or a single, large, centrally located neurofibril in the giant axons. No such structures have been observed in this material. The neurofibrils described here, having a uniform diameter of approximately 1000 Å and a complex internal structure, were found in all axons of the earthworm and in the cytoplasm of all neurons [15], where they accumulate preferentially in the axon hillock. Structures of similar shape, size and distribution have been observed in living spinal ganglia of chick embryos [21] and in fixed nerve with the electron microscope [2, 20, 33] and have been interpreted as mitochondria. It is not believed that the fibrils in the earthworm are mitochondria chiefly on the basis of their relationship to axon filaments and their internal structure which has no features comparable to the cristae mitochondriales [26], internal double membranes [37] or coaxial lamellae [3] which are characteristic of mitochondria in different cells.

Fig. 4. Higher magnification of Fig. 1 showing the "string of beads" arrangement in axoplasmic filaments. At *a* typical filament where particles are equidistant. At *b* the particles are crowded near one another on the filament. At *c* the particles appear disconnected but still retaining a linear arrangement. Osmic acid fixation. × 61,000.

Fig. 5. Neurofibrils from an axon in the neuropile showing a parallel bundle of axoplasmic filaments (arrow) continuous with the end of a neurofibril. Areas of low density are also seen along the fibrils. Osmic acid fixation. × 50,000.

Fig. 6. Preparation similar to that of Fig. 5 showing a dense strand of material forming a spiral in the interior of the neurofibrils. The end of a fibril frays out into axoplasmic filaments (arrow). × 50,000.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There is considerable argument in the literature as to whether the neurofibrils observed by the light microscope in fixed and even in living nerve cells are true entities. In living tissue culture cells of chick embryos Levi and Meyer [17] and Weiss and Wang [43] have demonstrated neurofibrils as continuous structures whereas Hughes [14] using the same tissue observed only round or rod-like granules which were arranged longitudinally in the processes. Living human sympathetic ganglion cells [22] and living rabbit neurosecretory cells [27] do not show neurofibrils. Examinations of fixed nerve cells and axons with the electron microscope favor the interpretation that neurofibrils are the result of lateral aggregation and condensation of axon filaments brought about by the fixative [4, 29, 31]. The results of this study agree with the first part of this interpretation, but do not support the belief that the aggregation of axon filaments is a result of fixation. In this material, neurofibrils appear to be the result of an orderly process yielding always the same structures, as judged by the uniformity of the neurofibrils encountered in all specimens studied. Also, there are areas, as the giant axons, where neurofibrils are consistently absent from most of the fiber and others, as in the preneuronal side of the septum, where they are invariably more numerous. Fixation artefacts could not possibly show a preferential distribution of this nature.

Neurofibrils possess considerable elasticity [17, 30]. The coiled structure observed here in their interior may possibly explain this property.

It was also found [5] that crab and lobster nerves shorten as a result of electrically induced impulse propagation and this was interpreted as the possible result of an active molecular reorganization of the axoplasm. This work indicates that the submicroscopic picture of nerve fibers may depend upon their physiological state at the instant of fixation. It is, therefore, not unreasonable to suggest that the formation of neurofibrils from axoplasmic filaments may be a reversible physiological process associated with some phase of nervous activity. Also that the composition of the medium of experi-

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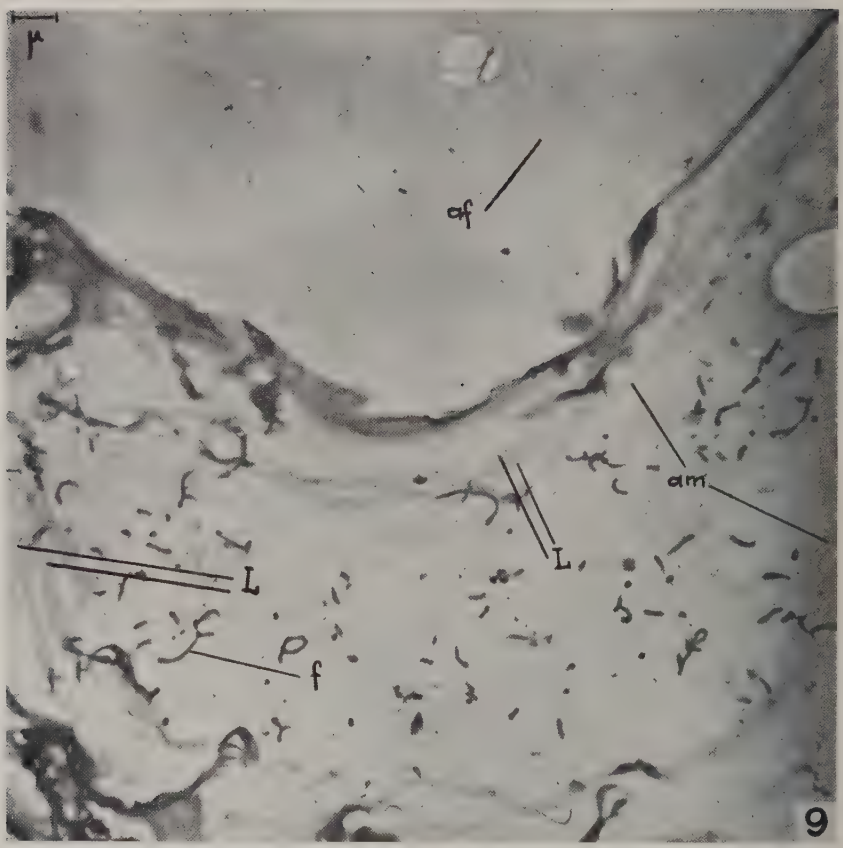
Fig. 7. A group of neurofibrils from a small axon of the neuropile. Bundle of axoplasmic filaments in left half of figure interpreted as a transitional stage in the formation of a neurofibril. Arrows point to pairs of neurofibrils twisted around each other. Osmic acid fixation. × 24,000.

Fig. 8. Montage of thirteen electron micrographs representing the entire cross section of a lateral giant axon at the level of a synapse. Arrow points to the oblique septum that separates the two overlapping synaptic members. Ribbon-like, membranous lamellae occupy the entire thickness of the sheath (*sh*). Similar lamellae are seen to form the septum, which originates in the sheath at *a* and *b*. The axon membranes (am_1 and am_2) are indicated. Note lack of neurofibrils from upper axoplasm; also cross-section of small neuropile axons (*ax*) and line of contact (*ct*) between lateral and median giant fibers. The sheath of the latter is visible on the far right side of the figure. Osmic acid fixation. × 2000.



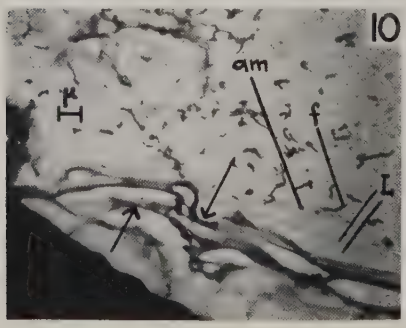


Fig. 9. Part of Fig. 8 under higher magnification showing the axolemma (*am*) to be identical in electron density and structure to the lamellae (*L*) of the sheath and septum; *af*, axoplasmic filaments; *f*, neurofibrils. Osmic acid fixation. × 6000.

Fig. 10. Longitudinal section through a synaptic septum in a median giant axon. The lamellae that have remained associated appear as dense black bands (arrows). At places where individual lamellae (*L*) peel off they appear identical to the axon membrane (*am*). Neurofibrils (*f*) are also seen. Osmic acid fixation. × 3000.

mental tissues would probably be an important factor determining the presence or absence of neurofibrils from these tissues.

Schultze [35] established for the first time that the sheath of annelid giant axons is myelinated, blackening with osmic acid. This was supported by other investigations [23] and also confirmed by birefringence studies [41]. The results of this investigation, when analyzed in the light of studies of myelin lamellae isolated from vertebrate nerve [11], support the view that the entire sheath of the earthworm giant axons consists of myelin lamellae, the innermost of which is the axon membrane.

All recent investigations of the synapse with the electron microscope [8, 10, 25, 28, 32, 36] agree to the fact that axo-axonal, axo-dendritic and axo-somatic synapses are characterized by the close apposition or contact of two plasma membranes contributed by each member of a pair. From the present observations this is also true of the synapse in the small axons of the earthworm which are covered by a single myelin-like membrane. In the giant axons, however, the synapses are characterized by the interposition of several myelin lamellae between pre- and postsynaptic axoplasms. Since these macrosynapses have been found to be very little delaying [7] compared to other synapses the large amount of myelin present at these sites not only lacks an insulating function, as it is generally believed for this substance, but seems to be expedient in some way to the passage of the nerve impulse.

It is accepted at the present that a thick myelin sheath increases the speed of conduction [42]. The variations in the speed of synaptic transmission, however, have not been associated with any histological entity. Further experiments are needed before the significance of the presence of myelin at the synaptic area can be evaluated.

SUMMARY

The ventral nerve cord of the earthworm has been studied with the electron microscope. Axoplasmic filaments having a "string of beads" appearance and a diameter of 165–200 Å were seen in all axons. Smooth neurofibrils of varying lengths and 0.1 μ thickness were also observed. They have a complex internal structure. Their nature and formation are discussed. The sheath of giant axons consists of layered myelin membranes. The small axons are invested by a single membrane which appears identical to the unit lamellae of myelin. At the synapse, in the giant axons, several myelin membranes separate pre- and postsynaptic axoplasm. In the small axons, however, only two membranes are present at the synapse.

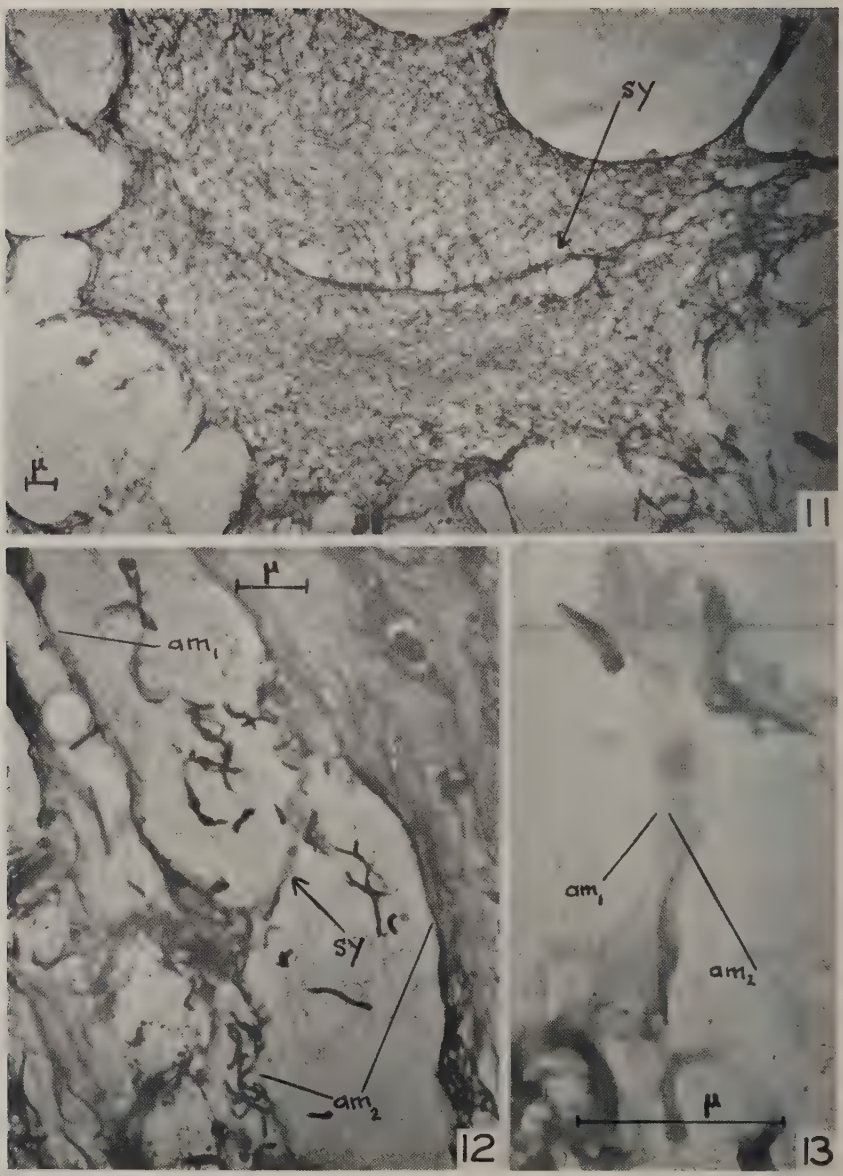

sy
μ
11
μ
am1
sy
am2
12
am1
am2
μ
13

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Fig. 11. Lateral giant axon at the level of a synaptic septum, which extends from the left to the right side of the figure. Note excessive precipitation of the formalin-fixed axoplasm into a dense network. The arrow (*sy*) points to a portion of the septum where several parallel lamellae may be resolved. × 4000.

Fig. 12. Longitudinal section of axons in the neuropile, showing a synapse (*sy*) in the largest one. The axon membranes (am_1, am_2) are also seen. Osmic acid fixation. × 10,000.

Fig. 13. Higher magnification of Fig. 12 showing the oblique septum to consist of the two axon membranes (am_1, am_2) running parallel at some distance from each other. Osmic acid fixation. × 30,000.

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MAST CELLS AND TISSUE WATER[1]

STUDIES ON LIVING CONNECTIVE TISSUE IN THE HAMSTER CHEEK POUCH

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Intraperitoneal injection of water into rats is followed by disruption of the mast cells in the mesentery [6]. This phenomenon has also been observed in ovomucoid-induced oedemas in the rat [4] and in human urticaria factitia [1]. Studying mast cells under the influence of water in the phase contrast microscope Zollinger [10] observed swelling of the cell body as well as of the individual cytoplasmic granules. At the same time there was a decrease in the metachromasia normally resulting from toluidine blue staining. Prolonged treatment with physiological saline produced similar changes of the mast cells. On injection of physiological saline and Tyrode solution into the peritoneal cavity of rats Fawcett [6] noted that most mast cells remained unaffected, but occasional cells released their granules.

Tissue sections treated with hyaluronidase do not show a subsidence of the metachromasia in the mast cells [9, 1]. After having injected hyaluronidase into the subcutaneous tissue of the hamster Compton [5] studied fixed connective tissue specimens. In other experiments, he studied unfixed tissue treated with hyaluronidase. He did not observe any alteration of the metachromasia of the mast cells upon staining with safranin. By contrast, Asboe-Hansen [2] observed degranulation of the mast cells in biopsy specimens of human, normal and myxoedematous, skin injected intracutaneously with hyaluronidase. The phenomenon was explained as cellular activity in restoration of a normal ground substance.

Since mast cells abound wherever new formation of connective tissue takes place [3], not least in oedemas during organization, and since they

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Table I

Influence of various fluids on living tissue mast cells.

Fluid	Changes in mast cells within 30 minutes
Sterile water	Degranulation Disruption Granule swelling
Physiological saline	Degranulation
Tyrode sol.	Degranulation
2 per cent saline sol.	Shrinkage
Hyaluronidase in physiological saline (100 and 1000 VRU/ml)*	Rapid degranulation
Hyaluronidase in Tyrode sol. (100 and 1000 VRU/ml)	Rapid degranulation
Hyaluronic acid 1 and 2 per cent in physiological saline	Insignificant changes
Traumatization (tissue oedema)	Degranulation Disruption Granule clumping

* Viscosity-reducing units.

appear to play a role in the formation of the connective-tissue ground substance [1]—which is a most important fluid depot—it seemed relevant to study the response of living connective-tissue mast cells to altered fluid conditions in the tissues.

Recently the authors showed that systemic application of histamine and chemical histamine—liberator substances caused widespread and marked degranulation of the mast cells in the connective tissue of the hamster cheek pouch [7].

MATERIAL AND METHODS

The test object was the hamster (*Mesocricetus auratus*) cheek pouch. The method, previously described [8], permits detailed microscopic study of the morphology of mast cells in *living* connective tissue with intact circulation—partly after staining with toluidine blue in physiological saline, 1:100 to 1:100,000, and partly in unstained connective tissue—in the bright field and phase contrast microscope, by means of water immersion objectives, and physiological saline as immersion fluid.

The present paper concerns the local effect of various fluids upon the connective-tissue mast cells. One of the two cheek pouches of the hamster was invariably used for control. Both cheek pouches may be drawn out simultaneously and each spread over a hole in a disc of cork which permits translumination. The fluids were injected between the two layers of the cheek pouch or dropped onto the lower membrane of the outspread, split pouch. The control fluid was allowed to act for an equal length of time on the contra-lateral cheek pouch of the same hamster. By dropping the fluid directly onto the denuded connective tissue, the traumatic effect of injecting is avoided to some extent. In order to approach physiological conditions as far as possible, the hamsters were placed on a warming stage mounted on the microscope.

The depolymerizing effect of hyaluronidase upon hyaluronic acid in the connective-tissue ground substance was utilized partly by injecting the connective tissue with the enzyme and partly by dropping a concentrated solution of testicular hyaluronidase[1] onto the denuded connective tissue of the divided cheek pouch. One of the preparations used contained 100 viscosity-reducing units per mg and another, highly concentrated one 3000 V.R.U./mg or about 27,000 V.R.U./mg N.

Hyaluronic acid, produced from bovine vitreous humour and from human umbilical cord[2] was injected—in 1 and 2 per cent solutions—into the connective tissue of the cheek pouch or dropped onto the connective-tissue aspect of the membrane.

To obtain a local tissue oedema by a method as physiological as possible, the cheek pouch was traumatized by rubbing it a few times between the thumb and index finger.

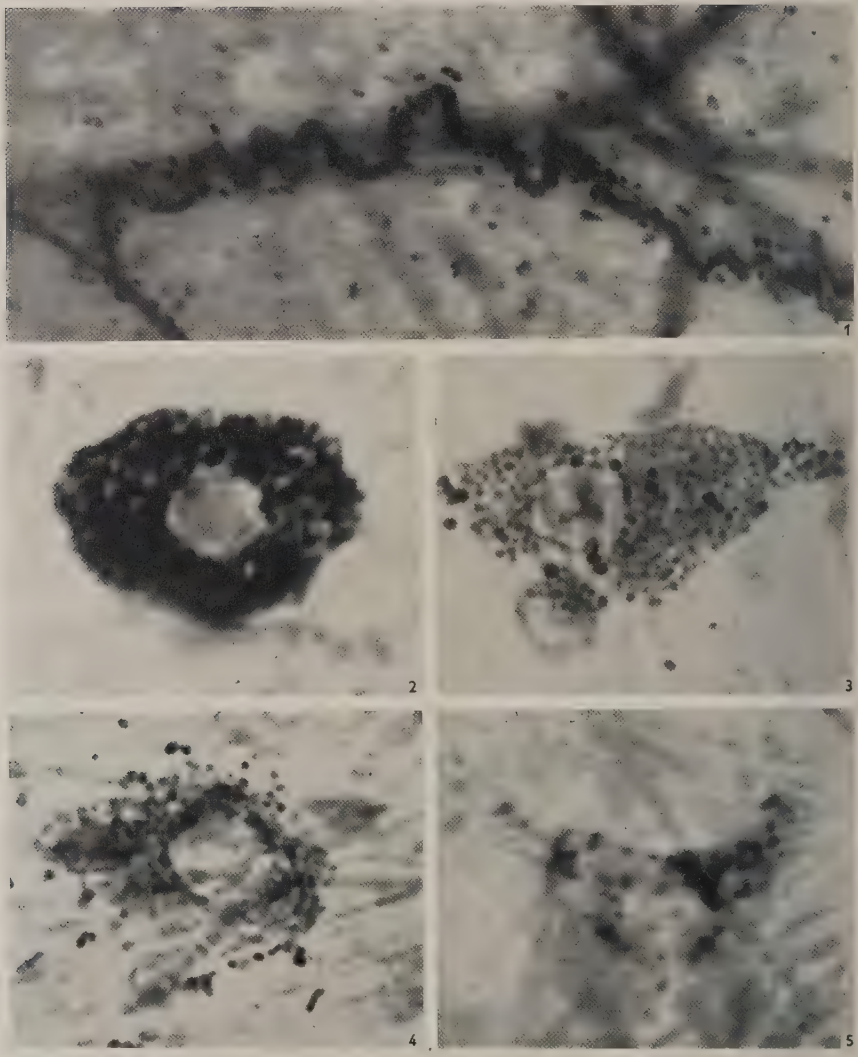
RESULTS

Injection of the fluids into the connective tissue of the cheek pouches invariably caused disruption, i.e. irreversible damage, of some mast cells. Perceptibly fewer disintegrated cells were seen after the dropping procedure.

After application of *water* a considerably larger number of cells in various stages of *degranulation* were observed than in the directly stained control pouches. This phenomenon apparently reflects an active granule release by the living cell. In some of the cells the granules appeared swollen. Many granules were found lying free in the connective-tissue ground substance after cell *disruption*, showing intense metachromasia; they retained their colour unchanged for hours (Fig. 4).

Varying numbers of *macrophages* containing in their cytoplasm metachromatic granules were observed, a phenomenon which was interpreted as evidence of phagocytosis of emitted mast-cell granules. These granules retained their metachromasia for some time within the cytoplasm of the phagocytizing cells (Fig. 5).

[1] Kindly supplied by A/B Leo, Hälsingborg, Sweden.
[2] Kindly supplied by Wyeth International Ltd., Philadelphia, Pa., U.S.A.



After treatment with *physiological saline* and *Tyrode solution* a considerable increase in the number of degranulated cells was observed as early as 20–30 minutes after application. These cells were fairly often surrounded by a zone of intercellular substance showing more intense metachromasia than more distant areas. Entirely degranulated cells showed a basic pattern like a honeycomb or sponge (cf. Fig. 3).

Thirty minutes after application of a *2 per cent saline solution* the mast cells revealed less degranulation than in the control pouches treated with physiological saline. The cells were smaller and their granular structure seemed to be denser.

Hyaluronidase caused marked degranulation occurring even within 10 minutes, at which time the control specimens still had well-granulated cells. Even in these specimens a few cells were well stained and metachromatic after a fairly long time of treatment. Similarly, the granules remaining in partially degranulated cells showed normal metachromasia (Fig. 3). The size of the cells was not definitely altered. No extracellular metachromasia was noticed, nor any signs of phagocytosis.

Hyaluronic acid, 1 and 2 per cent in physiological saline, did not induce any significant changes of the mast cells within 40 minutes. The cells stood out intensely metachromatic and densely granulated (Figs. 1, 2).

After *traumatization* of a cheek pouch mast cells with conglomerated granules and more or less degranulated were observed in considerably larger numbers than in the control pouch; in addition, a large number of disintegrated cells, as well as macrophages with phagocytized granules.

DISCUSSION

The disruption following upon application of water is probably due to osmosis. The extra-cellular granules originating from disrupted mast cells

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Fig. 1. Microphotograph of mast cells of living connective tissue stained with toluidine blue 1:40,000 in physiological saline and examined in incident light. Magnification approx. × 80. Notice accumulation around vessels.

Fig. 2. Normal, metachromatically granulated mast cell. Staining: toluidine blue 1:40,000 in physiological saline. Magnification × 2900.

Fig. 3. Almost completely degranulated mast cell influenced by hyaluronidase. Notice basic pattern like a honeycomb or a sponge. A few metachromatic granules (black) are left within the cell. Staining: toluidine blue 1:40,000 in physiological saline. Magnification × 2900.

Fig. 4. A mast cell of living tissue during disruption. Staining: toluidine blue 1:40,000 in physiological saline. Magnification × 2900.

Fig. 5. Macrophage with phagocytized mast cell granules in its cytoplasm. Staining: toluidine blue 1:40,000 in physiological saline. Magnification × 2900.

resisted the action of water. There seems to be reason to believe that the granule substance may come into the ground substance partly by a disruption caused by some violent—physical or chemical—action during which the cell perishes, and partly by physiological degranulation probably involving some alteration of the granules, e.g. with regard to water solubility, before or during the passage from within to outside the cell.

The few cells, which resisted prolonged influence of physiological saline, Tyrode fluid and hyaluronidase, may have been protected from this influence by adipose tissue, thicker, fibrous connective-tissue layers, etc.

An increase in the water content of the connective-tissue ground substance appears to entail a movement of the granule substance from the intra- to the extracellular space. The diffusion of the fluids in the tissues as well as the rate of hydration are evidently factors influencing the conditions in the experimental as well as the control specimens.

The rapid release of granules from the mast cells after application of hyaluronidase may be interpreted as a compensatory reaction to the topical situation established by a breakdown of the hyaluronic acid of the ground substance, the removal of the components by diffusion, and an increase in the diffusivity of water in the tissues. This view is supported by the fact that the cells are altered very slowly by the influence of a hyaluronic acid solution. The last-mentioned fluid is possibly the real control fluid, yielding a physiological environment for the mast cells.

The fact that mechanical trauma of the cheek pouch entails tissue oedema as well as degranulation of the mast cells, supports the interpretation of the degranulation as a physiological response to increased tissue water. It may be comparable to the degranulating effect of histamine and histamine liberators, which also cause tissue oedema.

The implications of degranulation are not fully understood. Previous experiments have brought much evidence that mast cells form mucinous ground-substance components of the hyaluronic acid type which represent a hydrated gel in the connective tissue. The present observations indicate that free water in the tissues is immediately converted into connective-tissue ground substance by a granule release. Any disturbance in the physicochemical balance between the mast cells and the intercellular matrix may immediately elicit a mast-cell response.

The perivascular accumulation of mast cells is consistent with the view that they are to some extent functionally linked to vascular permeability, being ever ready to change a perivascular oedema into a mucinous ground substance.

SUMMARY

Applied topically, water entails granule swelling and active degranulation of *living* mast cells of the master cheek pouch, and, to some extent, irreversible disruption. Physiological saline and Tyrode solution cause degranulation alone, 2 per cent saline solution shrinkage. Hyaluronidase brings about rapid degranulation, hyaluronic acid insignificant or very slow alterations of the mast cells. Mechanical trauma is followed by degranulation, granule clumping as well as disruption. Injection of the fluids provokes traumatization phenomena; dropping on the denuded connective tissue is more lenient and is preferred.

The authors advance the view that the mast cells contain and are able to release mucopolysaccharide material to the intercellular substance in response to tissue oedema. Free water in the tissues may be bound and converted into mucinous substance.

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MERCAPTOACETIC DERIVATIVES OF 1:4-NAPHTHOQUINONE AND OF 1:4-BENZOQUINONE IN THEIR ACTION ON THE MITOSIS OF CHICK FIBROBLASTS

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The chemical investigation of the interaction of mercaptoacetic acid with 1:4-naphthoquinone has shown that, under certain conditions, mainly the *bis*-mercaptoacetic acid derivative is formed [2]. Mercapto-DL-lactic acid and β-mercaptopropionic acid have given similar results. In the following paper the action of these new compounds on the mitosis of chick fibroblasts has been compared with the actvity of the known *mono*-mercaptoacetic derivatives [1].

By extending the addition of mercaptoacetic acid on 1:4-naphthoquinone-2:3-epoxide a new *mono*-mercaptoacetic derivative has been obtained which contains a hydroxylic group, vicinal to the thiolacetic residue. A compound of this structure has, so far, not been investigated in tissue cultures.

The results obtained by the addition of two molecules of thiolacetic acid to 1:4-naphthoquinone invited the study of a substance which contained more mercaptoacetic residues attached to a quinone. The known *tetra*-mercaptoacetic derivative of 1:4-benzoquinone has, therefore, been prepared and investigated in tissue cultures of chick fibroblasts.

METHODS

The methods used have been described previously [3, 6].

RESULTS

The results are shown in Table I. Formulas I–VII represent the following substances:

I S-(1:4-naphthoquinolyl-2) mercaptoacetic acid,
II S-(2 methyl-1:4-naphthoquinolyl-3) mercaptoacetic acid,
III S-(2-hydroxy-1:4-naphthoquinolyl-3) mercaptoactic acid,
IV S:S-(1:4-naphthoquinolyl-2:3) *bis*-mercaptoacetic acid,

V S:S-(1:4-naphthoquinolyl-2:3) *bis*-DL-lactic acid,
VI S:S-(1:4-naphthoquinolyl-2:3) *bis*-β-mercaptopropionic acid,
VII S:S:S:S-(1:4-benzoquinolyl-2:3:5:6)-*tetra*-mercaptoacetic acid.

Substances I and II have previously been prepared [1, 4] and examined in tissue cultures. Substance I has been reexamined simultaneously with Substance IV. Substances III, IV, V and VI are new crystalline compounds. Their preparation and their chemical behaviour will be described elsewhere. Substance VII has been synthesised by Schubert [7].

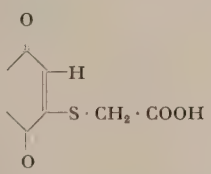
Table I demonstrates that of the three *mono*-MAA-derivatives of 1:4-naphthoquinone, Substance III gives the greatest inhibition of mitosis in cultures

TABLE I

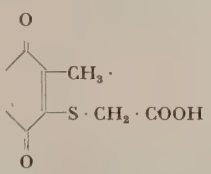
Inhibition and promotion of mitosis (M.I. and M. Pr.) by certain 1:4-quinolyl-mercaptoacetic acids.

(Mercaptoacetic acid: M.A.A.)

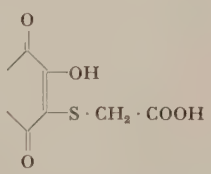
mono-M.A.A. derivatives of 1:4-naphthoquinone



I
M.I. 72 % at $10^{-5}M$

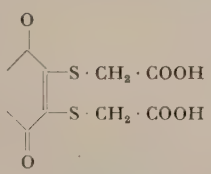


II
M.I. Nil at $10^{-5}M$

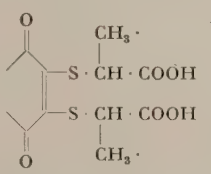


III
M.I. 87 % at $10^{-7}M$

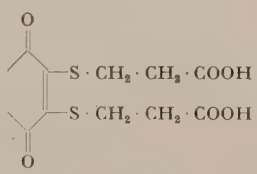
bis-M.A.A. derivatives of 1:4-naphthoquinone



IV
M.I. 39 % at $10^{-7}M$

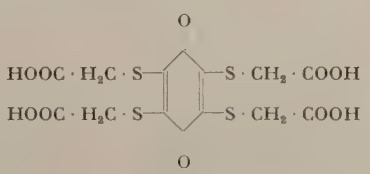


V
M.I. Nil at $10^{-5}M$



VI
M.I. Nil at $10^{-5}M$

tetra-M.A.A. derivative of 1:4-benzoquinone



VII
M. Pr. + 42, 67, 13, 34, 60 % at $10^{-6}M$

of chick fibroblasts. In this compound a hydroxylic group is vicinal to the MAA-residue. Its antimitotic activity is 100 times greater than that of Substance I.

In the series of the *bis*-mercaptoacetic derivatives only Substance IV is endowed with antimitotic activity. Substances V and VI are inactive even at $10^{-5}M$. Substance IV is approximately 100 times more active than Substance I, the corresponding *mono*-MAA derivative.

The influence of concentration of the antimitotic substances III and IV on the degree of mitotic inhibition is shown in Table II.

TABLE II

Mitotic inhibition of substances III and IV at different concentrations in percentage of the controls.

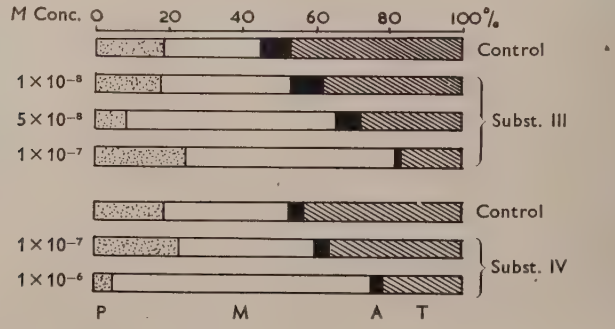
Substance III		Substance IV	
M conc.	Mitotic inhibition, %	*M* conc.	Mitotic inhibition, %
1×10^{-7}	89	1×10^{-6}	73
5×10^{-8}	57	1×10^{-7}	39
1×10^{-8}	0	5×10^{-8}	0

It may be noted that occasionally numerically irregular results are obtained.

The phase distribution after application of Substances III and IV at different concentrations is shown diagrammatically in Table III.

TABLE III

Phase distribution after application of Substance III and IV at different concentrations. P, M, A, T stands for Prophase, Metaphase, Anaphase and Telophase respectively.



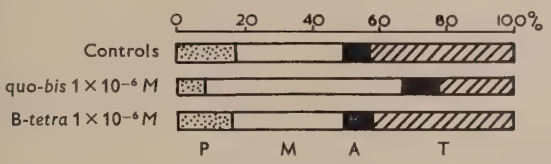
It will be seen from Table III that Substance III as well as Substance IV induce accumulation of cells in metaphase. With Substance III the accumulation appears already at $5 \times 10^{-6}M$, whilst Substance IV needs a concentration of $10^{-6}M$ to produce this effect. At this concentration cell oedema and a slight effect on resting cells (slight cell oedema, resting cells disconnected and rounded up, some exploded cells) are observed.

The action of the *tetra*-MAA-derivative in tissue cultures (Substance VII) is in contrast to the mitotic inhibition recorded with *mono*- and *bis*-MAA-derivatives of 1:4-naphthoquinone. This quinone has no antimitotic activity but increases the mitotic count considerably. The area of outgrowth is not affected by the application of the substance to the growing tissue. The substance gives normal phase distribution and no abnormalities have been observed at $10^{-6}M$. Even at $10^{-7}M$, there was a 35 per cent increase of the mitotic count. The mitotic count showed considerable variation at $10^{-6}M$; the concentration of $10^{-7}M$ has not been further investigated.

Table IV gives the phase distribution produced by the *tetra*-MAA-derivative of 1:4-benzoquinone compared with that produced by the *bis*-MAA-derivative of 1:4-naphthoquinone.

TABLE IV

Comparison of the phase distributions obtained with substances III and VII.



The quantitative results of the tissue culture experiment are given in Table V.

DISCUSSION

In the following discussion, the *mono*-MAA-derivatives of 1:4-naphthoquinone are called quo-*mo*, the *bis*-mercaptoderivatives quo-*bis*, and the *tetra*-mercaptoacetic acid derivative of 1:4-benzoquinone B-*tetra*.

In the quo-*mo* series, substance III of Table I (the 2-hydroxy-3-mercaptoacetic acid derivative of 1:4-naphthoquinone) shows a very high antimitotic activity (87 per cent mitotic inhibition at $10^{-7}M$). It is the strongest mitotic

TABLE V

Tissue cultures: Chicken fibroblasts, hanging drop method, 4th passage, six 24-hour cultures, fixed in Susa, stained in Heidenhain's haematoxylin.

Exp.	Molar conc.	Mitoses as % of mitoses of controls	Per cent inhibition	Phase distribution in % of total mitoses			
				Prophase	Metaphase	Anaphase	Telophase
		S-1:4-*naphthoquinolyl*-2) *mercaptoacetic acid* (*I*) *S*-(2 *methyl*-1:4-*naphthoquinolyl*-3) *mercaptoacetic acid* (*II*) (1673 *mitotic cells investigated*)					
1	Controls	—	—	23.1	38.1	2.4	36.4
2	1×10^{-5}	27.6 ± 2.3	72.4	15.1	35.4	16.0	33.5
3	1×10^{-5}	97.0 ± 8.8	3.0	21.7	36.0	3.0	37.7
		S-(2-*hydroxy*-1:4-*naphthoquinolyl*-3) *mercaptoacetic acid* (*III*) (4624 *mitotic cells investigated*)					
1	Controls	—	—	19.2	34.1	3.7	43.0
2	1×10^{-6}	no mitoses		—	—	—	—
3	1×10^{-7}	13.0 ± 22.4	87.0	24.7	57.1	1.3	16.9
4	Controls	—	—	19.1	26.3	8.2	46.4
5	5×10^{-8}	42.8 ± 1.4	57.2	8.7	56.5	7.4	27.4
6	2×10^{-8}	100.0 ± 2.6	0.0	19.0	37.2	7.5	36.2
7	1×10^{-8}	98.2 ± 3.0	1.8	18.6	35.0	7.8	38.6
		S:*S*-(1:4-*naphthoquinolyl*-2:3) *bis-mercaptoacetic acid* (*IV*) (5424 *mitotic cells investigated*)					
2	Controls	—	—	19.2	34.1	3.7	43.0
2	1×10^{-6}	27.3 ± 4.5	72.7	4.9	69.9	3.7	31.5
3	1×10^{-7}	61.0 ± 9.4	39.0	22.9	37.9	3.6	35.9
4	Controls	—	—	17.5	32.8	6.4	43.3
5	5×10^{-7}	63.1 ± 6.3	36.9	13.1	39.2	7.5	40.2
6	2×10^{-7}	87.8 ± 1.7	12.2	12.3	38.2	7.7	41.8
7	5×10^{-8}	100 ± 1.8	0.0	13.3	40.6	6.5	39.6
		S:*S*-(1:4-*naphthoquinolyl*-2:3) *bis*-DL-*lactic acid* (*V*) (6698 *mitotic cells investigated*)					
1	Controls	—	—	21.9	29.6	7.2	41.3
2	1×10^{-5}	100 ± 4.4	0.0	19.4	29.5	6.5	44.6
3	1×10^{-6}	100 ± 3.4	0.0	21.5	28.8	6.4	43.3
4	Controls	—	—	14.8	35.7	8.2	41.3
5	1×10^{-5}	101 ± 2.4	0.0	17.6	35.1	7.1	40.3
		S:*S*-(1:4-*naphthoquinolyl*-2:3) *bis*-β-*mercaptopropionic acid* (*VI*) (4564 *mitotic cells investigated*)					
1	Controls	—	—	20.0	32.9	4.6	42.5
2	1×10^{-5}	110 ± 4.9	0.0	21.1	33.1	4.7	41.1
3	Controls	—	—	15.8	33.8	5.5	44.9
4	1×10^{-5}	118 ± 6.1	0.0	16.9	34.2	7.3	41.6

Table V (*continued*).

Exp.	Molar. conc.	Mitosis as % of mitoses of controls	Per cent increase	Phase distribution in % of total mitoses			
				Prophase	Metaphase	Anaphase	Telophase
		S:S:S:S-(1:4-*benzoquinolyl*-1:2:3:5:6)-*tetra-mercaptoacetic acid* (*VII*) (27,825 *mitotic cells investigated*)					
1	Controls	—	—	11.4	41.8	3.3	43.5
2	1×10^{-5}	127 ±5.2	27.0	14.3	40.0	5.7	40.0
3	1×10^{-6}	142 ±6.2	42.0	17.6	34.5	6.8	41.1
4	1×10^{-7}	135 ±4.2	35.0	19.1	36.1	5.8	39.0
5	Controls	—	—	14.8	33.2	8.6	43.4
6	1×10^{-6}	167 ±4.7	67.0	12.5	34.2	11.1	42.1
7	Controls	—	—	19.1	30.0	4.5	46.4
8	1×10^{-6}	112 ±4.6	12.0	20.9	28.6	3.4	47.1
9	Controls	—	—	18.7	29.7	4.9	46.7
10	1×10^{-6}	160 ±5.1	60.0	18.8	26.0	9.4	45.8
11	Controls	—	—	17.4	30.0	7.8	44.8
12	1×10^{-6}	113 ±3.8	13.0	16.3	35.1	6.7	41.9
13	Controls	—	—	20.2	34.5	5.1	40.8
14	1×10^{-6}	134 ±7.1	34.0	23.0	33.1	4.2	39.7
15	Controls	—	—	15.3	32.3	7.9	44.5
16	1×10^{-6}	143.5 ±7.3	43.5	14.5	31.5	7.2	46.8

The areas of outgrowth of the cultures in Experiment VII (5 and 6) were measured by planimetry of outline drawn with a camera lucida with linear magnification 6.1.
6 Controls; growth area in mm²: 1595m 266 ±6.6 per cent.
6 Comp. VII; growth area in mm²: 1780m 296 ±10.0 per cent.
Comp. VII as per cent of Controls: 11.6 ±13.4 per cent.* (*Not significant.)

inhibitor in the group of hydroxylated 1:4-naphthoquinone derivatives encountered so far. 5-hydroxy-1:4-naphthoquinone (Juglone) is active as a mitotic inhibitor to 51 per cent at $6 \times 10^{-6} M$ and the 2-hydroxy-1:4-naphthoquinone to 53 per cent at $10^{-5} M$ [6].

The high antimitotic activity of Substance IV in the quo-*bis* series has some interest. This compound contains 2-thiolacetic residues and arises from the interaction of an excess of thiolacetic acid with 1:4-naphthoquinone in neutral solution. When a quinone is added to growing cells it encounters an excess of thiol compounds in neutral solution.

In contrast, the *mono*-thiolacetic derivative is obtained in acid solution, an excess of the thiol compound being avoided. As the main –SH compound in embryo extract is glutathione the question arises whether the results

obtained with mercaptoacetic acid can be applied to glutathione. The experiment dealing with this problem will be reported in due course, and only one point will be mentioned now. The incresae of antimitotic activity from the *mono*-substituted Substance I to the *bis*-substituted Substance IV is brought about by a mechanism which is fundamentally different from that by which Substance I produces its antimitotic effect. This will be shown in a subsequent paper.

The *bis*-DL-mercaptoacetic acid and the *bis*-β-mercaptopropionic acid derivative of 1:4-naphthoquinone do not inhibit mitosis. With the *bis*-mercaptopropionic acid derivative (Substance IV) a new feature appears as this substance seems to increase the mitotic count. The figures are statistically of doubtful significance, however, and it remains to be seen whether *bis* substituted quinones which contain longer chains than those of β-mercaptopropionic acid, will give clearer results.

The greatest increase of the mitotic count is produced by the B-*tetra* compound (Substance VII). The mechanism of its action is now being studied.

Summarising the results, we see that the addition of one molecule of thiolacetic acid to 1:4- naphthoquinone produces a substance with weak antimitotic activity (Substance I); the addition of two molecules of mercaptoacetic acid gives a powerful mitotic inhibitor (Substance IV). Other mercaptoacid adducts not only fail to exert this effect (Substance V) but seem to indicate a reverse action changing the mitotic inhibition into a promotion of mitosis (Substance VI). The incorporation of four molecules of thiolacetic acid into benzoquinone brings the increase of the mitotic count to a maximum (Substance VII).

SUMMARY

1. When added to tissue cultures of chick fibroblasts, the compounds resulting from the addition of thiolacetic acid to 1:4-quinones give different results according to the number of thiolacetic residues incorporated in the quinone.

(*a*) Addition of 1 mol. thiolacetic acid to 1:4-naphthoquinone produces a weak mitotic inhibitor.

(*b*) Addition of 2 mol. thiolacetic acid to 1:4-naphthoquinone results in a compound with strong antimitotic properties.

(*c*) Addition of 4 mol. thiolacetic acid to 1:4-benzoquinone gives a substance which shows no mitotic inhibition but increases considerably the mitotic count.

2. After addition of mercapto-DL-lactic acid and of β-mercaptopropionic acid to 1:4-naphthoquinone no mitotic inhibition has been observed.

3. The addition of thiolacetic acid to 1:4-naphthoquinone-2:3-epoxide has produced the strongest hydroxylated antimitotic agent so far obtained.

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THE SARCOLEMMA AND FIBROUS ENVELOPE OF STRIATED MUSCLES IN BEEF[1, 2]

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Barer [2] in his comprehensive review of the structure of the muscle fiber opened his discussion on the sarcolemma with these words: "Considerable confusion exists regarding the nature of the investing membrane of the striated muscle fiber... but the exact localization of this membrane in the muscle cells is a matter for conjecture." Since then, due largely to the advent of the electron microscope [4, 8, 9, 10], especially the work of Rozsa, Szent-Györgyi and Wyckoff [9], great strides have been made towards a far better understanding than ever realized hitherto, of the structural composition of the muscle fiber membrane. The present study employed a simple, direct and easy technique with which to demonstrate the true sarcolemma and a closely applied fibrous sheath that envelopes the muscle fiber immediately outside the sarcolemma. The methods partly involved fixation and staining of muscle tissues not because of unawareness of the undesirability of such procedures [2] but rather that they were deemed necessary specifically for showing the latter component of the sarcolemmous membrane. No digestion by enzymes, production of retraction clots, swelling of muscle substance by means of weak alkaloids or acids, or any other like means were used.

[1] This paper was presented before the Sixty-ninth Annual Meeting of the American Association of Anatomists, Milwaukee, Wisconsin, April 5, 1956 [11].

[2] Journal Paper No. 129, American Meat Institute Foundation.

(Figs. 1–14, *in toto* preparations; figs. 15 and 16, celloidin sections.)

Fig. 1. Intact muscle fibers isolated from fresh, raw unfixed beef *Longissimus dorsi*, stained with hematoxylin and Van Gieson's. Note numerous nuclei. Magnification × 80.

Fig. 2. A row of empty muscle fiber envelopes from raw beef *Semitendinosus* fixed in Zenker-formol and stained with Pollak's trichrome. Note anastomosing collagenous fibers of endomysial origin attached to them. Magnification × 150.

Fig. 3. One large intact muscle fiber with adjoining muscle fiber envelopes from cooked beef *Semitendinosus* fixed with Zenker-formol, unstained. Dark field illumination. Magnification × 320.

Fig. 4. Muscle fiber envelopes from raw beef *Semitendinosus*, unfixed and unstained. Magnification × 320.

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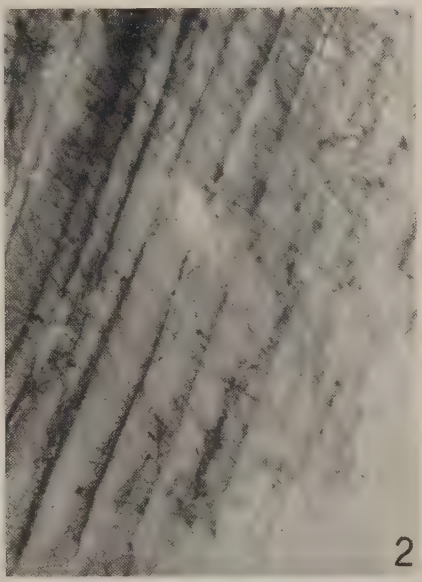
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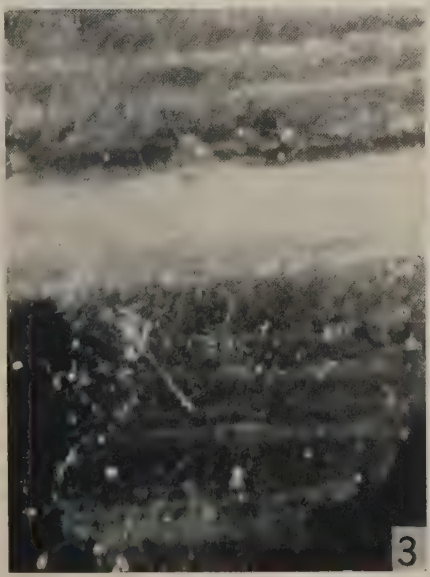
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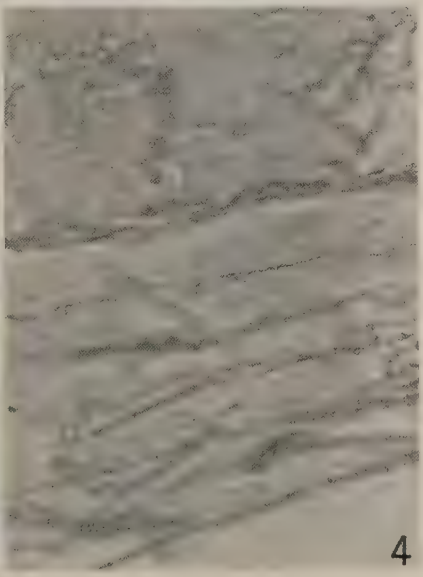
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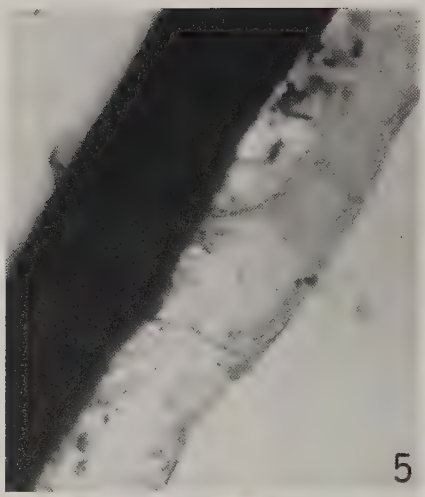
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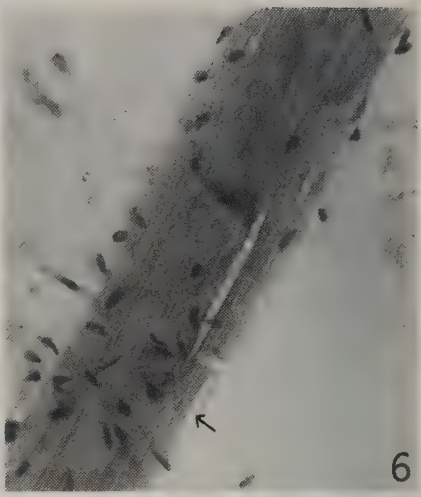
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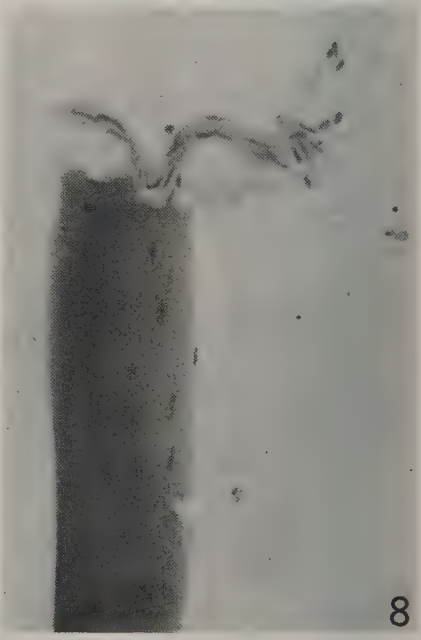
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MATERIALS AND METHODS

The technique utilized a Waring blendor modifed in such a way that the resulting isolated materials from a small piece of muscle tissue were undamaged and structurally intact. For example, single muscle fibers completely freed of their connections were obtained in this way for the determination of muscle fiber extensibility [12]. The necessary modifications were as follows: The blades of its cutter were made dull by filing. With the knife so dulled, raw or cooked muscle samples previously fixed in Zenker-formol were blendored from 2 to 5 seconds at regular speed (approx. 10,000 rpm). For raw unfixed tissues, the dulled knife was used, but in addition the blendor was run for 5 seconds at a reduced speed (approx. 3300 rpm) by inserting into the circuit a 200-watt light bulb serially. All samples were longitudinal strips of beef *Longissimus dorsi* and *Semitendinosus* muscles measuring approximately 2 · 3 cm and 3 mm in thickness. For each operation, the blendor was filled with water, in which the sample was immersed, just above the blendor knife.

Such a procedure yielded single muscle fibers and connective tissue fragments: some of the latter were identified as "empty envelopes" of the muscle fibers. In certain instances segments of muscle fibers with ends still held together by the sarcolemma were obtained. In either case, the materials can be studied to advantage *in toto* without staining, or stained in Pollak's trichrome. Also, 10 μ celloidin sections of the same muscles cooked (broiled) to an internal temperature of 150°F [13] were prepared and stained with hematoxylin and Van Gieson's mixture. They served to verify certain features shown by the other preparations.

RESULTS

Materials demonstrating the fibrous envelopes of muscle fibers were obtained from samples which had been cooked and/or fixed in Zenker-formol, but never from raw, unfixed muscles. Some of them were stained with Pollak's trichrome, cleared in xylol and mounted in balsam. Others were unstained preparations. Fig. 3, photographed with dark-ground illumination,

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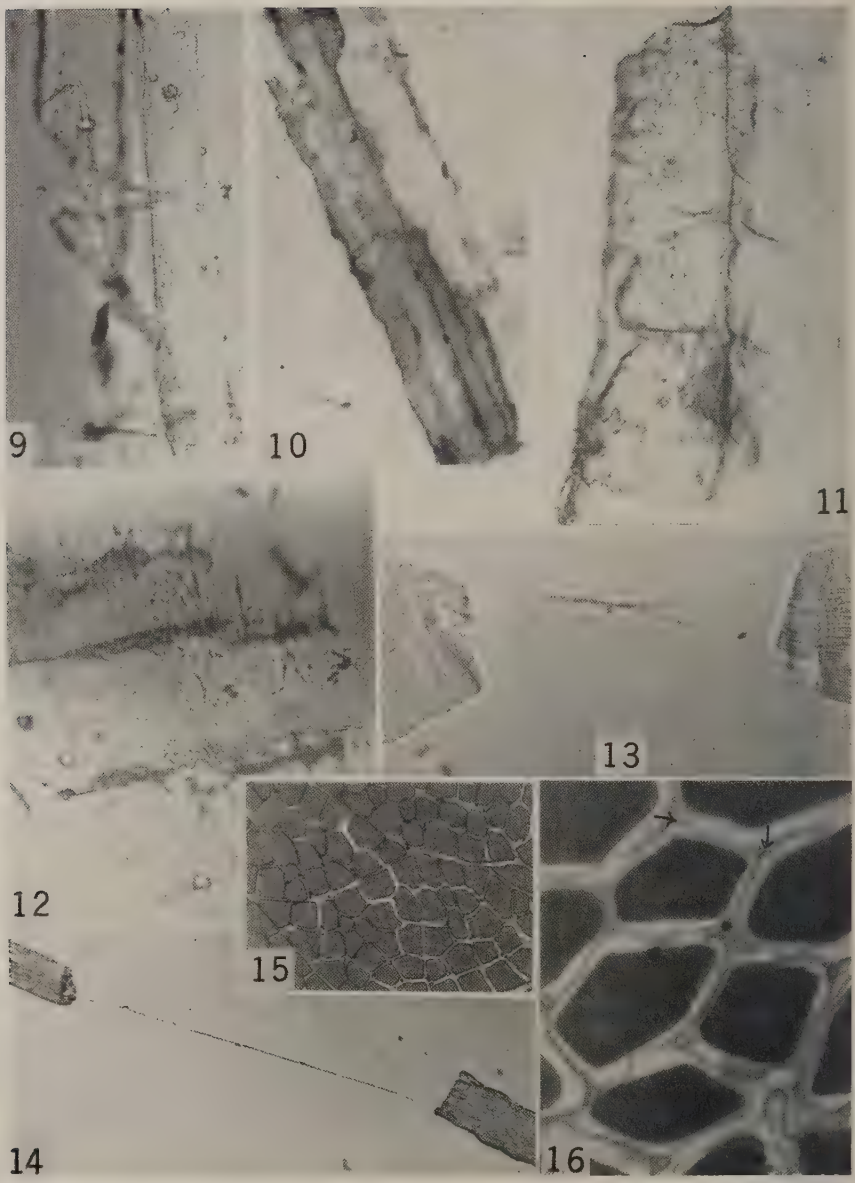
Fig. 5. One intact muscle fiber (left) and one empty muscle fiber envelope, somewhat damaged at places, from beef *Semitendinosus*, fixed in Zenker-formol and stained with Pollak's trichrome. Magnification × 450.

Fig. 6. One single muscle fiber, from raw unfixed beef *Longissimus dorsi*, enclosed in its envelope. Most of the nuclei shown are those of connective tissue cells closely associated with the muscle fiber envelope. Triangle points to a capillary showing the nucleus of an endothelial cell. Individual myofibrils are clearly visible. Hematoxylin and Van Gieson's stain. Magnification × 650.

Fig. 7. A single muscle fiber from cooked beef *Longissimus dorsi*, in the process of losing its envelope in the direction of the arrow. The triangle marks the boundary between the bare portion of the fiber (near bottom of figure) and that still retaining the envelope. Hematoxylin and Van Gieson's stain. Magnification × 450.

Fig. 8. A single muscle fiber from fresh, raw, unfixed beef *Longissimus dorsi*, showing a rare occurrence; the shedding of the muscle fiber envelope. The shed envelope (toward top of figure) had collapsed into a tubular sheath, carrying with it many nuclei of the connective tissue cells. Hematoxylin and Van Gieson's stain. Magnification × 575.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is an example of the latter kind. It shows one intact muscle fiber with rows of empty envelopes on either side. Most of the irregular spots seen in the photomicrograph were debris; the tiny spherical dots of more or less uniform size, on the other hand, reflect the structural composition of the envelope under consideration. Similar envelopes (empty) seen under conventional illumination are illustrated in Fig. 4. These structures were cylindrical in shape, most of them were broken (cf. Fig. 5) during blendoring while their contents, bare muscle fibers, escaped. The lack of a uniformly sharp focus in Fig. 4 was caused by a considerable diameter of these envelopes and possibly also the fact that at places there may be more than one layer of these structures involved. At any rate, they represent only the muscle fiber envelopes, not associated in any way with the connective tissues of the endomysium. For a contrast, a row of similar muscle fiber envelopes attached to definitive endomysial collagen fibers (stained blue-green with Pollak's trichrome) is presented in Fig. 2.

The muscle fiber envelope revealed in this study is believed to be of a fibrous nature even though their actual composition is too small to bee seen under the light microscope. The incorporation of fibers into them is indicated by the numerous interstices, particularly well brought out in Figs. 3, 9, 11 and 12. The dotted or porous appearance so portrayed appeared to be the result of interweaving of the fine fibers, somewhat comparable to the threads in a fabric. A few stained fibers in Figs. 11 and 12, as those shown in Fig. 2, were definitely collagen fibers of the endomysium. The fibers shown in

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Fig. 9. One muscle fiber envelope showing attached capillaries, from cooked beef *Longissimus dorsi*, fixed in Zenker-formol and stained with Pollak's trichrome. Magnification × 275.
Fig. 10. Intact and broken muscle fiber envelopes from cooked beef *Longissimus dorsi*, unfixed and stained with Pollak's trichrome. Magnification × 275.
Fig. 11. Two muscle fiber envelopes from beef *Semitendinosus*, fixed in Zenker-formol and stained with Pollak's trichrome showing the attachment of some endomysial collagenous fibers along the line of union. Magnification × 560.
Fig. 12. Three muscle fiber envelopes from cooked beef *Longissimus dorsi*, fixed in Zenker-formol and stained with Pollak's thrichrome. Magnification × 560.
Fig. 13. Ends of two segments of a single muscle fiber linked by its sarcolemma, the latter forming a double-cone after fusion in the middle; from raw unfixed beef *Semitendinosus*, unstained. Magnification × 780.
Fig. 14. Another view of sarcolemma connecting the ends of two segments (further pulled apart than the one shown in Fig. 13) of a muscle fiber from raw unfixed beef *Semitendinosus*, unstained. Magnification × 225.
Fig. 15. Transverse section of raw beef *Longissimus dorsi* fixed in Zenker-formol and stained with hematoxylin and Van Gieson's showing normal structures. Magnification × 80.
Fig. 16. Transverse section of cooked beef *Longissimus dorsi* fixed in Zenker-formol and stained with hematoxylin and Van Gieson's, showing shrunk muscle fibers and merged neighboring muscle fiber envelopes with little endomysial space (arrows) left. Magnification × 560.

Fig. 11 were oriented in a way distinct from those in Figs. 2 or 12; both, however, indicate part of the intricate system of the endomysial connective tissues in striated muscles. Aside from that, Figs. 2 and 11 also portray the topographical relationship between the muscle fiber membrane and the adjoining endomysial connective tissue fibers.

In Fig. 9 is shown a single muscle fiber envelope with attached capillaries. That these envelopes are actually empty shells, much like a snake's shed skin, is illustrated in Fig. 10. One of the envelopes in this figure was broken, leaving behind only a shadow or "ghost" while the others are, for the most part, intact. A capillary coming in sharply at a 90° angle is seen attached to one of them. The well indicated nuclei were those of endothelial cells of the capillary wall and free connective tissue cells (mostly fibrocytes) of the endomysium.

Significantly, the materials considered so far, were all obtained from samples which were cooked, chemically fixed or both. Fig. 16 presents the typical structure of this kind of muscle tissue seen in cross section. Comparing with that of the normal structure of raw muscle (Fig. 15) quickly reveals what cooking (broiling) had done to the muscle fibers and their membranes. Unquestionably, it had caused a pronounced shrinkage of the muscle fiber protein or actomyosin [10] as a direct result of moisture loss. This differential shrinkage, in turn, caused the muscle fiber envelopes to be separated from the shrunk actomyosin as clearly indicated in Fig. 16. Equally well indicated, was the merging and actual fusion of the separated muscle fiber envelopes from the adjacent muscle fibers except along corners. The endomysial spaces (arrows) became thus confined to areas enclosed by the unfused portions of the separated muscle fiber envelopes. Consequently, the sizable spaces between the muscle fibers are not endomysial spaces but rather spaces that resulted from the separation of the actomyosin from the fiber envelopes. These observations would help to explain why the suspension obtained from blendoring fixed and cooked muscle tissues consisted of "bare" muscle fibers and individual or rows of empty muscle fiber envelopes as just described.

Occasionally, however, such a separation may not occur. Fig. 5, for instance, illustrates one intact muscle fiber side by side with another; all that was left of the latter is its "shell", i.e. an empty envelope. The latter, similar to those shown in Fig. 11, indicated irregular spots of breakage from which the muscle fiber presumably had been thrown out during blendoring. Sometimes, the separation may be incomplete as the case illustrated in Fig. 7. The muscle fiber was isolated from a cooked sample and was shown partially

enclosed in its envelope. The situtation affords a comparison between the appearance of a bare muscle fiber (toward bottom of Fig. 7) and portions still enclosed in its envelope. This rare instance constitutes positive demonstration for the discrete membrane that envelopes a muscle fiber, and incidentally it also made possible an estimation of the thickness of this membrane. For such a calculation, allowance is made for the fact that a portion of the envelope was actually turned back on itself so that the measured thickness represented twice the thickness of the muscle fiber envelope. This value was determined to be approximately 1.6 μ. The nuclei shown in this case are of special interest. It is easy to distinguish those of the muscle fiber, which are invariably oriented with their longer axes in the direction of the fiber (cf. Fig. 8) from those that belong to the membranes. The latter, besides being much more numerous, have a random orientation. Note also that no endomysial connective tissue fibers of any kind are present in Fig. 7.

Blendoring of raw, unfixed muscle tissue at the reduced speed yielded quite different materials. Mixed in the suspension were segments of muscle fibers and small pieces of connective tissues. Most of the latter, however, were pulverized and damaged to a point that they could serve no useful purpose in this study. A large majority of the isolated muscle fibers were intact, i.e. enclosed in their envelopes (Fig. 6). The fiber shown in Fig. 6 was more highly magnified and also better focused than the one in Fig. 7, hence it clearly shows the myofibrils with cross striations. The presence of numerous nuclei oriented exactly like those attached to the covered portion of the muscle fiber shown in Fig. 7 proves that it is an intact muscle fiber. The strip at the point of an arrow (Fig. 6) is believed to be actually the envelope. On this assumption, its calculated thickness is approximately 1.5 μ. This thickness is approximately equivalent to the thickness of two myofibrils and is in close agreement with the value calculated from Fig. 7. Note a looped capillary carrying the nucleus of an endothelial cell, applied to the surface of the muscle fiber (cf. Figs. 9 and 10).

Among the muscle fibers from raw unfixed samples only rarely one or two fibers may be found to be devoid of its envelope. However, attempts were made to rid a fiber of its sheath by manipulation with two pairs of forceps under the binocular dissecting microscope. The result of one successful trial is illustrated in Fig. 8. It shows a muscle fiber after it had been bared of its envelope. The latter had collapsed, but can be recognized without difficulty by virtue of the attached nuclei. This shows very definitely that the muscle fiber envelopes under consideration are highly elastic fibrous sheaths and that the connective tissue cells closely associated with it are virtually insepara-

ble from them and therefore may be considered as being an integral part of the envelope. There are reasons to believe that the sarcolemma proper was detached together with the envelope.

Only in exceptional cases were muscle fibers found which were broken in such a manner that the two ends remained firmly connected. By use of the dissecting and compound microscopes the broken segments were shown to be held together by an ultrathin colorless and structureless membrane, which was barely visible under optimal illumination. By pulling the two fragments of the fibers apart with forceps, the membrane collapsed in the middle at a point roughly equi-distant from the ends of the fragments (Fig. 13), forming a double-cone. Some idea of the thinness of this membrane may be gained from the fact that the cones were mostly invisible except along their edges. An estimate of its thickness was made by comparing it with the myofibrils which were distinctly shown in this photomicrograph. The myofibrils measured under oil immersion ranged from 0.5 to 0.8 μ in diameter. It is evident that the membrane under consideration is only a fraction of the thickness of a myofibril. It is in the order of 0.1 μ. This value agrees with the thickness of sarcolemma reported by Barer and Jones [7, 8]. It is to be remembered that these workers used muscles other than beef, hence some slight discrepancy in the estimated thickness of sarcolemma may be due to species and/or muscle differences.

By pulling the broken fiber ends farther apart, the double-cone continued to elongate until it broke when its fused portion reached a length of 2 mm or more (Fig. 14). It is not ascertained whether the increased length of the cone involved new parts of the sarcolemma from the broken fiber ends or due to the elasticity of the already-exposed sarcolemma.

DISCUSSION

In 1947 Long [5] described the sarcolemma as a double structure consisting of an inner plasma membrane and an outer delicate sheath of reticular fibrils closely adherent to the former, and favored restricting the true sarcolemma to that thin, structureless membrane. This bipartite nature of the muscle fiber membrane is a view shared by others [2, 8, 9] including the present writer. There is, however, no unanimity of opinion regarding the structural kinship of the two membranes in question. For instance, Rozsa, Szent-Györgyi and Wyckoff [9] stated that "the threads apparently are not an integral part of the body of the membrane, but are attached to its surface and form part of a system of threads surrounding the muscle fibers" (p. 203). Some

clarification of this situation is needed. The question now arises: Is the fibrous envelope (membrane or sheath) such as the one shown in the present study and frequently mentioned by others a part of the sarcolemma, or does it belong to the endomysial connective tissue system?

Certain observations related above appear to have some bearing on this question. First, muscle fibers isolated from raw unfixed muscle tissue were mostly whole muscle fibers complete with their sarcolemma and the immediate fibrous envelope (see Fig. 6). The latter exhibited thereby a greater structural affinity with the sarcolemma proper than with the endomysium. Second, when muscle tissue was denatured by cooking or/and chemical fixation, both membranes become detached from the actomyosin so that upon blendoring, bare muscle fibers deprived of any membrane were consistently obtained, and concurrently the two muscle fiber membranes invariably stayed together as empty muscle fiber envelopes (see Figs. 5 and 7). Thus, the affinity between the two membranes remained even after the slight denaturation wrought by cooking. Third, rarely but true, the sarcolemma and its envelope could be made to shed together from an undenatured muscle fiber (Fig. 8). The only time the sarcolemma proper was shown alone, i.e., not accompanied by the fibrous envelope was when two segments of a broken muscle fiber were seen connected by the sarcolemma (Fig. 13). But such a case was rarely encountered. These observations give sufficient ground for supporting the bipartite view of Long [5] and Barer [2]. On this basis, it seems reasonable that the inner and outer membranes should be designated, respectively, as the sarcolemma proper (or true sarcolemma) and the sarcolemmous envelope or sheath. The word, sarcolemma, when used alone, should refer to both of these structures compositely.

Due to the limiting resolving power of the light microscope, this work revealed no structural details of the sarcolemma proper. As demonstrated, it was very thin and homogeneous, probably non-fibrillar and structureless as maintained by other investigators [2, 4, 7, 8]. However, a certain physical property of the sarcolemma proper was demonstrated, namely, it was stretchable though not truly elastic (see Fig. 14).

The structural nature of the sarcolemmous envelope has not yet been satisfactorily defined. From the informative electron micrographs of Rozsa, Szent-Györgyi and Wyckoff [9] it is definitely of a fibrous nature. This seemed well reflected by the presence of numerous interstices on the envelopes seen under the light microscope (see Fig. 9, 11 and 12). Bairta [1] and Meyer and Bernfeld [6] thought that the fibers comprising the envelope were probably collagen. Our results agreed with Barer [2] and Conte and Pieser [3]

that they were not collagenous for the reason that the envelopes considered here failed to take any stain (Pollak's trichrome). Both Reed and Rudall [8] and Rozsa, Szent-Györgyi and Wyckoff [9] described fibers that seemed most definitely collagen; but these are undoubtedly similar to the anastomosing endomysial collagenous fibers such as seen in Fig. 2. The presence of two kinds of fibers [9] on the basis of their striations (periodicity) provided a clue that the material used in their study could happen to be bordering on the junction of the fibrous sarcolemmous envelope and the endomysium. It would seem likely that the fibers showing the interrupted striations were endomysial collagen while those that were continuously striated belonged to the envelope. Whether or not the latter type is reticular as proposed by Long [5] remains to be determined.

Possible functional importance of the two components of the sarcolemma, particularly the outer fibrous one, have been adequately discussed [2, 8, 9].

The technique used in this study, crude as it may seem, has nevertheless proved its value in demonstrating structures of the muscle fiber membranes that are supplementary to the details revealed by electron micrographs [9]. Of particular significance is the fact that this study has disclosed a structural relationship of the fibrous sarcolemmous envelope with the sarcolemma proper on the one hand and with the endomysium on the other. Finally, the description of these structures in a species (bovine) not usually employed in this field of research perhaps also deserves attention.

SUMMARY

A Waring blendor with dulled knife and reduced speed was used for isolating muscle fibers and connective tissues from small samples of beef muscles. Significantly, the sarcolemma proper, a thin, homogeneous, non-fibrous membrane about 0.1 μ thick was found linking the ends of broken muscle fibers from raw unfixed tissue only. From cooked or/and chemically fixed samples, the isolated muscle fibers were "bare", i.e. deprived of their membranes, the latter being found in the form of empty tubular envelopes either singly or in groups. The separation between the muscle fiber substance (actomyosin) and the surrounding membranes, so observed, is the result of a differential protein denaturation brought about by the cooking and fixing processes as evidenced by the structures of cooked muscle tissues seen in 10 μ celloidin sections. No structural details were obtained from the muscle fiber envelopes on account of the limited resolving power of the light microscope, but judging from their characteristic appearance of having numerous

tiny interstices, they are probably fibrous, though not likely of the collagenous type in the light of the recent results obtained with electron microscopy.

The results of this work have confirmed the bipartite nature of the muscle fiber membrane held by many investigators in this field, and have advanced reasons for believing that the true sarcolemma and the outer fibrous envelope are truly components of an intact muscle fiber membrane. It is suggested on these grounds that the term sarcolemma, when used alone, may refer to the composite membrane of a muscle fiber as a whole, whereas the two components should be known, respectively, as the sarcolemma proper (or true sarcolemma) and the sarcolemmous envelope.

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CELL GROWTH IN NORMAL AND SYNCHRONOUSLY DIVIDING MASS CULTURES OF *TETRAHYMENA PYRIFORMIS*[1]

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By means of controlled temperature shifts we have synchronized cell division of *Tetrahymena pyriformis* GL. [18, 19, 24]. While in a normal exponentially growing culture the division index is in the range of 0.05–0.10, it rises in the first "synchronous" division—after suppression of division activity for several hours—to about 0.85. From this and the fact that during our standard treatment the mean cell size, determined by volume estimation, weighings on the diver balance [18] and nitrogen determinations [23], increases by a factor 3–4, it is evident that we have to some extent dissociated two processes, normally of predominant importance in the life of cells: growth and cell division.

The effect of temperature changes on the processes leading to cell division in single cells were further investigated by Thormar in this laboratory [21]. The present study is concerned with growth as reflected in the volume of the individual cells. At several well defined points of the established curve for population growth [3] the individual volumes of a constant number of cells were measured and the size distribution curves were constructed.

Volume distribution studies were made by several authors on protozoa [1, 7, 10] and nuclei of tissue culture cells [4, 6, 20] and revealed consistent results under reproducible conditions. If these environmental conditions are altered, however, the cell size may change to a considerable extent. Weis found that the volume of starving cells of *Tetrahymena pyriformis* S. was reduced to 9.1 per cent of the original. This phenomenon could be reversed completely by addition of suitable nutrients [22].

The mean cell size is constant under standard conditions and changes drastically if the environment is altered. Furthermore, the size distribution of a given sample will be shown to be characteristic for different phases of

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population growth. For this reason we felt that, besides cell counts and division index, an investigation concerning the relation of the distribution of the individual cell sizes to some average size would be a sensitive tool for the study of growth. From the size distribution curves we get quantitative information on the influence of the temperature treatment on different parts of the reproductive cycle. In this connection a general problem arises: Is it possible from the "static" picture of the distribution of size or other cell constituents (e.g. DNA) measured on each cell (of one sample) to gain information on the "dynamic" picture of size change or of synthesis of the constituents in question within the cell cycle [17]?

MATERIAL AND METHODS

Organism.—The organism used throughout the experiments was *Tetrahymena pyriformis* GL., grown under sterile conditions in a 2 per cent (w/v) proteose peptone medium, enriched with 1 per mille (w/v) liver fraction and salts as described in a preceding paper [19]. Each experiment was carried out on cell populations from 4.5×10^3 cells per ml at the inoculation level up to 10^6 cells per ml in the maximum stationary phase. The cells were grown in 150 ml culture medium, which was thoroughly shaken by elliptical rotatory movements of the 450 ml Fernbach culture flasks to ensure proper aeration and random distribution of the cells in the medium. The flasks were submerged in a water bath, the temperature of which was automatically controlled and changed according to the experimental requirements.

Inoculation.—Stock cultures were grown as previously described [19]. For the inoculation of the experimental flask, 3 ml of a three day old stock culture with about 2.3×10^5 cells per ml was used.

Cell counts.—The estimation of the population density was based on direct counts as described elsewhere [15].

Cell size and pH.—In the course of the experiments there was a change in pH from 6.6 after inoculation to 8.0 in the maximum stationary phase. However, the pH was almost constant for the first 15 hours of the experiments in which the mean size of the cells was reduced to 50 per cent. Later, when the pH changed, the mean cell size remained almost constant. The distribution of the cell volumes was apparently not affected either, since at the different pH levels mentioned, the shape of the size distribution curve was identical. No influence of the pH of the culture medium on the mean cell size and the distribution of the cell volumes was observed.

Estimation of the cell volume.—The cells were fixed in Bouin's fluid after the prescription given by Romeis [14]. This fixative proved useful already for the careful studies by Loefer and Corliss [5, 9]. In preliminary experiments the volume change of twenty individual cells, representing different stages of the cell cycle was followed as a function of time after the addition of the fixative. The percentual shrinkage obtained after the elapse of a given time was the same in all cells. The shrinkage is most rapid after the addition of the fixative and levels off in time. Observations on 100 cells showed a volume reduction of 10 per cent after 1 hour and 12.5 per cent in the

course of 4 hours, as compared to the volume measurement 3 minutes after the addition of the fixative. Photographs were always taken after 1 hour. A Leitz Ortholux Microscope and a Leica camera were used (magnification 10 × 10, film: Ilford Pan F). Photographs of a Leitz-stage micrometer supplied a scale for absolute size. On the copies the linear dimensions of the fixed cells were enlarged 270–300 times. Both cell diameters were measured in millimeters, converted to microns and the volume calculated after the formula for an ellipsoid of rotation around the long axis: $V = \frac{4}{3} a^2 b$, in which a is the minor and b the major semiaxis. A simple slide rule of paper strips was constructed in order to adjust both measured millimeter values on one side and to read off the actual figures for "$\frac{4}{3} a^2$" and "b" in microns, the multiplication of which resulted in the estimated cell volume in μ^3. In this manner the volumes of more than 6000 cells were calculated.

EXPERIMENTS AND RESULTS

Growth steps chosen.—The characteristic growth curves were established for normal and "heat-treated" growth (Fig. 1). Four experiments for the former and three experiments for the latter were carried out under reproducible standard conditions [19]. Each point represents the counting of 650 cells on the average. Test tube cultures were used for the inoculation, resulting in shortest possible initial lag, less than two hours.

Samples for cell counts and for size distribution studies were removed almost simultaneously from the experimental flasks. Nine characteristic growth steps were selected for normal growth and seven such steps in cultures subjected to our standard heat treatment. The standard temperature cycle applied was 28.5°C⇌33.9°C with switches from one level to the other every 30 minutes. Eight periods of elevated temperature were applied. The steps chosen in normal cultures were (Fig. 1): the inoculum (○ 1); at the end of the initial stationary phase, by which time there is an abrupt shift to the log phase (○ 2); four hours after inoculation, when the division index is twice that in later logarithmic growth (○ 3). This "hump" of division stages deserves some attention since it resembles a synchronized system, reported on by Browning, Brittain and Bergendahl [2]. It will be analysed elsewhere [16]. Two further steps at different levels within the logarithmic phase (○ 4 and ○ 5) were sampled. In the transitory phase from logarithmic growth to the stationary phase, the so called negative growth acceleration phase, two steps were selected, a few hours apart (○ 6 and ○ 7). In the early maximum stationary phase one step was chosen (○ 8), another about eighteen hours later (○ 9).

During and after heat treatment the culture was sampled at the following stages: after three heat shocks, when the division index is reduced practically to zero (△ 2); prior to the end of treatment (△ 3); prior to the first division

maximum (△ 4); after the first division (△ 5); and at two later points of growth (△ 6 and △ 7).

Preliminary information on the type of distribution.—Cell size distributions for the selected growth steps are presented in conventional histograms,

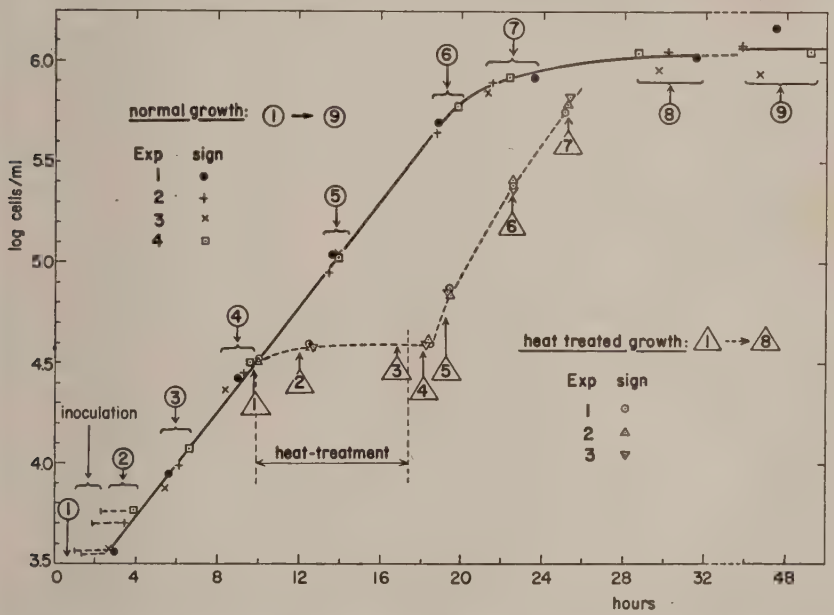

Fig. 1. Growth curves for normal and heat-treated cultures. The numbers in the circles indicate times for removal of samples for distribution studies of a normal culture; numbers in triangles apply similarly to the heat treated growth.

representing two typical experiments, one for a normal, and another one for a heat treated culture (Fig. 2). Each histogram is based on measurements of 100 cells. The volumes of these cells were grouped into classes, each ranging over $5 \times 10^3 \mu^3$. For instance the 50-line in Fig. 2 indicates the boundary between two classes, ranging from 46–50 and 51–55 $\times 10^3 \mu^3$ respectively.

Looking at the histograms of Fig. 2 one gets the impression that very obvious changes in average cell size and in absolute size variation occur in all phases of growth. The variation of the sizes in number ○ 1 is slightly more pronounced in number ○ 2; from there on in normal growth the absolute variation becomes progressively smaller, beginning in the logarithmic phase of growth up to the stationary phase (○ 3 through ○ 9). Parallel with this

phenomenon goes a progressive reduction of the mean size, which continues in early stationary phase, where there is ample oxygen supply. A culture in the stationary phase, however, which was grown in test tubes, not shaken and probably oxygen deficient, shows higher values for mean size and also a

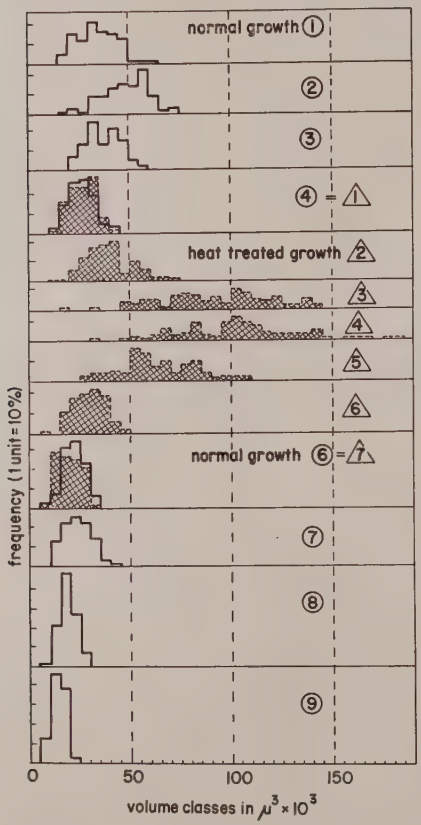

Fig. 2. Histogram for cell size distribution in two experiments in normal and heat treated cultures. The numbers in circles and triangles refer to the corresponding steps in Fig. 1. Frequency is plotted against volume classes on arithmetric scale.

larger variation. Such stock cultures were used for inoculation of the experimental cultures. It was not realized at that time that there existed such size differences in test tube cultures and aerated cultures.

In the course of heat treatment the range of cell sizes increases from $40 \times 10^3\mu^3$ in number △ 1 to $150 \times 10^3\mu^3$ in △ 3; this coincides with a considerable increase in mean size. After release from the heat treatment the mean size increases slightly before the first division, but the range is retained

(number △ 4). In the course of the successive division steps the range and mean cell size are reduced to values similar to the control (○ 6 = △ 7).

Pooling and analysis of the experiments.—The histograms supplied us with preliminary information within a single experiment. Parallel experiments, however, should be pooled in such a manner that the characteristic features of the distribution are emphasized and chance variations minimized. To meet these requirements, the probit method was applied for the further analysis of the data obtained experimentally. Thus the two parameters of a normal distribution, its mean ξ and standard deviation σ can easily be estimated graphically by M_0 and s_0, respectively.

From a biological and statistical point of view it has proved to be very useful to employ a logarithmic scale on the abscissa for the volume classes, as discussed in detail and stressed by Bucher [4].

The curve for a normal distribution is expressed as a straight line in a probit diagram. Consistent deviations from this straight line can be accepted as describing a true variation from the normal distribution. The data for four experiments in which cells were grown under normal conditions (at 29°C) without exposure to heat treatment were plotted as probit diagrams, shown in Fig. 3. Each of the four experiments is assigned a different symbol. Each curve is based on the measurement of 400 cells (100 in each experiment). The probit 5.00 divides the curve in a lower and upper part, each comprising 50 per cent of the observed cell number. It supplies us, therefore, with an estimate of the mean volume (M_0), read off on the corresponding point on the abscissa, since the median is identical with the mean in a perfect normal distribution. The slope of the curve gives an estimate of the standard deviation (s_0), which is read off as the difference between two probability units on the ordinate.

Since four experiments are pooled, it is essential to know how much the mean volume differs in the parallel steps of growth. Statistical analysis was therefore carried out on some samples which are close to the normal type of distribution. The v^2 test revealed that the four samples underlying each curve in the growth steps ○ 1, ○ 4, ○ 5 showed no significant difference. These parallel samples can therefore be considered as being taken from one experimental flask. At the growth steps ○ 8 and ○ 9, however, differences have developed between the parallel cultures. The variance ratio v^2 is 2.8 ($f_N = 3$, $f_D = 396$, $P = 2.5 - 5$ per cent) in number ○ 8 and 35.5 ($f_N = 3$, $f_D = 396$, $P < 0.05$ per cent) in number ○ 9 respectively.[1]

[1] f_N stands for the number of degrees of freedom in the numerator and f_D for the number of degrees of freedom in the denominator.

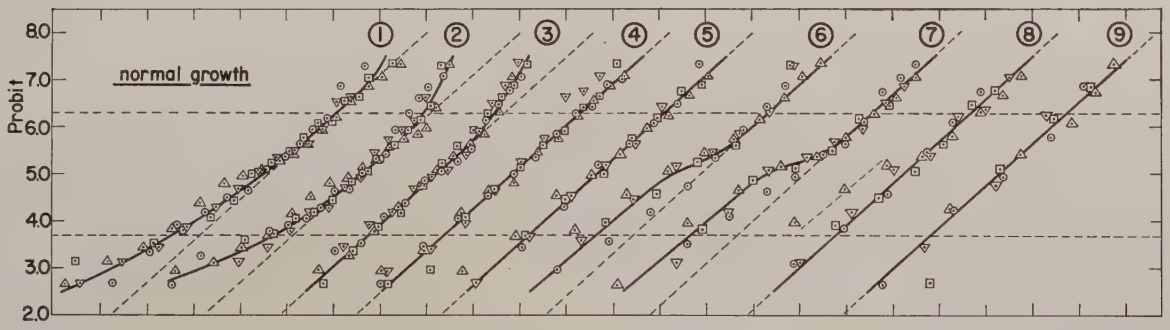

Fig. 3. Normal growth: probit diagrams of four parallel experiments. The numbers refer to the corresponding growth steps in Fig. 1.

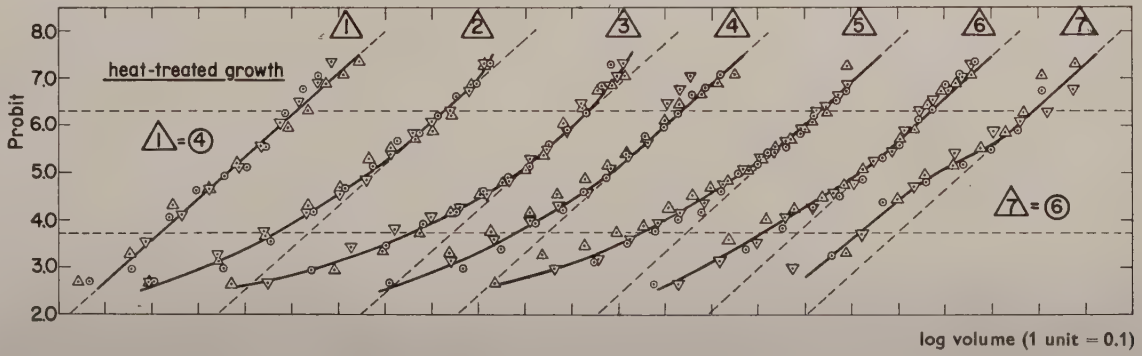

Fig. 4. Heat-treated cultures: Probit diagrams of three parallel experiments. The numbers refer to the corresponding growth steps in Fig. 1.

The probit diagrams were derived in the following way: The volumes were divided into classes, each ranging over $3 \times 10^3 \mu^3$. Going from the lowest to the highest class, the frequencies with which cells appear in the classes were cumulated by adding one class at a time for every step. The corresponding probit value was read in a table and plotted against the logarithm of the upper limit of each class. On separate probit diagrams each M_0 value was read off and the curves for the parallel experiments translated to a common point for M_0. As a matter of fact, the trend of points covered each other as shown in Fig. 3 and Fig. 4.

In this manner, the above mentioned steps ○ 1 through ○ 9 (see Fig. 1) of normal growth were pooled from four parallel experiments. The results are shown in Fig. 3. For reasons of simplification the actual figures of the logarithmic volume classes on the abscissa were omitted; the relative position of the curves on the abscissa is arbitrary and the curves are spaced in such a way that they keep apart. The dotted lines indicate the lower and upper 10 per cent limits, i.e. 80 per cent of the observed values are located in between.

It is apparent that all the curves have a common "backbone", indicated by the same slope of a straight line. This slope gives an estimate of the standard deviation of the distribution for logarithmic cell size in the case of normal growth (○ 4, ○ 5, ○ 9, Fig. 3). In heat treated growth the upper parts of the probit diagrams all have the same slope, but the probit curves bent off downwards to the left, indicating a long tail of relatively smaller cells (Fig.4). How this departure from normal distribution should be interpreted will be discussed below.

The results of three parallel experiments in synchronized cultures were calculated in the same way as just described for normal growth and the probit diagrams are shown in Fig. 4. Curve △ 1 is a control. It represents the size distribution typical of the stage ○ 4 in untreated logarithmically growing cultures. Curves △ 2, △ 3 and △ 4, derived from samples drawn from cultures exposed to intermittent heat treatment, show pronounced deviation from the straight line which has the same slope as the control. The computed standard deviations are, therefore, larger than the corresponding slopes at probit 5. As the standard deviation of the logarithmic size distribution is approximately proportional to the coefficient of variation we may state that the coefficient of variation in a case like △ 3, Fig. 4, would -if computed —be considerably larger than corresponding to the tangent which has the same slope as for normal growth.

While the histograms of Fig. 2 give an impression of the drastic changes

which occur in the course of normal and synchronized growth, the probit analysis reveals common features inherent in all cases, normal or heat-treated cell populations. First of all, the probit diagrams show that the three to four parallel samples on which each curve was computed may be consid-

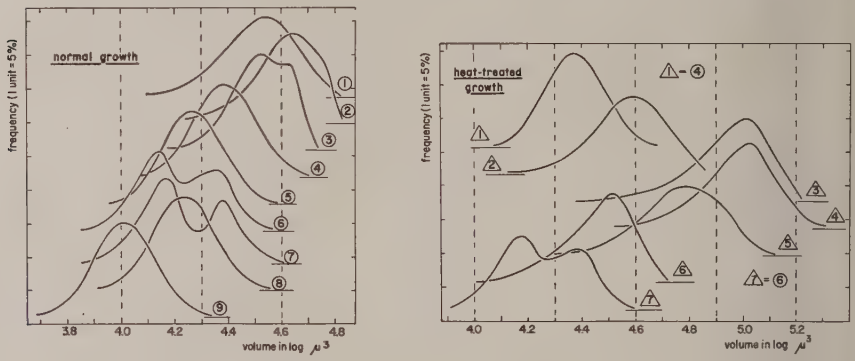

Fig. 5 (*left*). Normal cultures: Frequency curves for the different growth steps in Fig. 1. Difference between two parallel vertical dotted lines indicate doubling of volume. Frequency plotted against volume classes on a logarithmic scale. ○ 1, inoculum; ○ 2, after elapse of initial lag phase; ○ 3, ○ 4, ○ 5, during logarithmic growth; ○6, ○ 7, in negative growth acceleration phase; ○ 8, ○ 9, in maximum stationary phase.
Fig. 6 (*right*). Heat-treated cultures: Frequency curves for the different growth steps in Fig. 1. Difference between two parallel vertical dotted lines indicate doubling of volume. Frequency plotted against volume classes on a logarithmic scale. △ 1, prior to heat treatment; △ 2, △ 3, during heat treatment; △ 4, △ 5, △ 6 and △ 7, after heat treatment.

ered as identical. Furthermore, these distributions can easily be compared on the basis of the interpreted "backbone" features. These properties of the distributions would not seem to be easily discovered by means of histograms only, even if supplemented by computation of ordinary descriptive statistical parameters (mean, standard deviation, skewness etc.).

Size distribution.—In the two sets of probit diagrams of Figs. 3 and 4 continuous curves were adjusted to the points plotted. These were converted to frequency distributions (Figs. 5 and 6). For the sake of comparison, cells in division were separately measured, but not added in one of these distributions shown. The frequency of the cell number on the ordinate is plotted against the logarithm of the volume of the corresponding class. The ordinate is given in arbitrary units thus permitting displacement of the curves. The base-line of each curve is indicated below the number of each curve. Each unit on the ordinate equals 5 per cent in cell number. We shall return to these curves in the following discussion.

DISCUSSION

Normal growth.—Curve ○ 1 in Fig. 5 shows a size distribution for cells taken from the inoculum. This curve is not symmetric, but is tailing off to the left, indicating a relatively large proportion of small cells, clearly seen in curve ○ 1 of Fig. 3. These cells may have arisen in at least two ways differing in principle. They could have been formed by fission of cells twice as big as those considered. It will be seen from Fig. 3, curve ○ 1, and Fig. 5, curve ○ 1, that by the time of division, these cells must have been smaller than the size range, represented by the majority of all cells in the culture. Another, perhaps more likely possibility is that the development of anoxia in a growing test tube culture progressively inhibited cell division more than it inhibited growth, thus changing a population distribution, represented by curve ○ 4 into a curve of type ○ 1 of Fig. 5. In other words, the small cells were more sensitive to oxygen lack than the bigger cells and were therefore "left behind" in growth, thus producing the left tail of the distribution curve.

The cell size distribution at the end of the initial stationary phase (curve ○ 2, Fig. 5) indicates that all cells except the biggest have grown. The curve is therefore truncated to the right.

By the time sample ○ 3 was taken, the culture showed the same multiplication rate as before and after. However, the division index is relatively high [16, 2, 24]. Obviously, therefore the high division index does not go parallel with a high rate of multiplication. An obvious possibility is, that the average time span taken for cell division is increased. It will be shown elsewhere that this might be the case [16]. However, the cells not only remain longer in the visual part of fission, but also the preceding predivision phase appears to be elongated. This is reflected in a "hump" on the right part of the curve ○ 3. The culture is at a density (Fig. 1), where the population has doubled once after inoculation suggesting that every cell has divided once, therefore, this "piling" of division stages does not imply that these cells are "phased" in metabolic respects, but indicates that a definite phase of the cell cycle is elongated. When we come to stage ○ 4, Fig. 5, the piled cells have divided and the effect is faded away.

Numbers ○ 4 and ○ 5 show normal distributions in later stages of exponential multiplication.

At the end of the logarithmic growth phase the mean cell size remains the same in these well aerated cultures. It may become larger when oxygen is the only growth limiting factor (test tube cultures, Ormsbee's observation).

In numbers ○ 6 and ○ 7 the distributions show two maxima in the negative

growth acceleration phase. Inspection of Fig. 3 reveals that the four different cultures which are represented with different symbols differ more from each other in stage ○ 6 than later; then they become more alike in number ○ 7. The probit diagram of number ○ 7 (Fig. 3) shows that the upper part of the curve, forming the group with the bigger cells constitute 40 per cent (probit 5.25) of the whole sample. If these cells divide once, the whole population count must increase 40 per cent. This checks well with the actual increase in cell number to be read from Fig. 1. From stage ○ 7 to the maximum stationary phase (stages ○ 8) there is 45 per cent increase in the cell number of the culture. The following conclusion is therefore proposed: in the negative growth acceleration phase a rather rapid metabolic shift, occurring within five hours, from maximum multiplication to almost no multiplication is reflected in the cell size distribution: two distinct groups are formed, one comprising the smaller cells which are considerably slowed down in growth after division and on the other hand, a group of bigger cells which are slowed down in their preparation for division, but finally succeed in proliferation and "join" the smaller group with the result that we find again later (○ 8) the same distribution as in the logarithmic growth phase.

The development from stage ○ 8 to stage ○ 9 may reflect proportional size reduction in the single cells which have depleted the growth medium and cannot divide any more.

Heat-treated growth.—In the course of the applied standard temperature cycles, the shape of the individual cells changes gradually from the "pyriform" type to a more spherelike appearance. This is expressed in a quantitative relationship of both cell diameters: the mean width/length index is 0.60 in the control and becomes 0.78 after treatment. The frequency distributions (Fig. 6) reveal a fourfold increase of cell volume in the modal classes during treatment, viz.: from $24 \times 10^3 \mu^3$ in curve △ 1, which is a control to $106 \times 10^3 \mu^3$ in curve △ 3. Already after three heat cycles the size distribution of the cells shows the typical tailed left side (△ 2, Fig. 6). This phenomenon becomes more marked as the treatment continues. In that half of the population which consists of the smaller cells, the percentage volume increase is the smaller the smaller the cell is. The same holds true for the biggest cells, comprising about 5 per cent of the population (△ 3). Curves △ 4 and △ 5, Fig. 6, show two samples, taken prior to and after the first synchronous division, respectively. Five hours after the end of heat treatment the size has regulated back to normal mean (curve △ 6, Fig. 6) and the size distribution becomes almost identical with the untreated control after the elapse of two more hours (curve △ 7 in Fig. 6).

These changes of the distributions are also well illustrated by the probit diagrams in Fig. 4. For the sake of comparison a stippled line was drawn together with each curve, representing the slope of the normal distribution of curve △ 1. The tailed left side of the distributions of Fig. 6 is clearly seen as deviation from this stippled line.

These results show that temperature has a differential effect on growth, depending on the size of the cells and their position in the cell cycle. There is some evidence that the rate of cellular growth is faster after than before a division [12, 13]. If the inhibitory effect of heat is proportional to the rate of synthesis one would, therefore, expect that growth would be more inhibited in the small than in the big cells. It is possible that not only the absolute, but also the percentage rate of synthesis becomes smaller in the small cells than in the big ones. The data obtained may be interpreted that the percentage rate of synthesis in the small cells is inferior as compared to the big cells, as mentioned above.

It is remarkable that the distribution curves for cells cultivated under probable oxygen limitation and cells exposed to heat treatment are comparable as far as growth is concerned. Whether or not they are comparable in respect to cell division is a different matter and will be the subject of further studies [16].

SUMMARY

Tetrahymena pyriformis GL. was used for a study of growth based on distribution of cell size in mass cultures at optimal temperature (29°C) and after intermittent temperature shocks inducing synchronous division. Samples were drawn at well defined stages of growth (Fig. 1). Cell size was estimated from the measurement of the major and minor cell axes. Division stages were considered separately. Volume classes are plotted in an equidistant scale in Fig. 2, and on log scale in Figs. 5 and 6. A 3-day-old test tube culture was always used as an inoculum. The cells from the inoculum are fairly big and not quite normally distributed with respect to size. They become normally distributed in the course of few hours of logarithmic growth and remain so up to the time of the negative growth acceleration phase. Development of nutrient deficiency tends to split the population into two parts which become united in the maximum stationary phase again. In eight generations of logarithmic growth the mean cell size is reduced about 50 per cent. During heat treatment the mean cell size is increased about 300 per cent. In the course of synchronous division steps, the mean cell size is reduced after 5 hours to the size which is found in the control cultures. The considerable

change in mean cell size is not paralleled by striking changes of the coefficient of variation of the size distribution of the individual cells. Further analysis with the probit method revealed that the heat treatment produces a population with a distribution of cell sizes which deviates in a typical manner from normality. On a percentage basis, the small cells grow less than the big cells during heat treatment. The analysis of cell size distribution reveals similar reactions in test tube cultures and in cells exposed to temperature shocks in a well aerated culture.

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A COMPARISON OF THE INFLUENCE OF LOCALLY APPLIED THYROXINE UPON MAUTHNER'S CELL AND ADJACENT NEURONS[1]

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Recently attention has been directed to the role of thyroid hormone in the control of growth and differentiation of neurons in the amphibian brain. Tusques [20, 21], Hill [5], Baffoni and Catte [2, 3], Kollros *et al.* [7], Kollros and Pepernik [12], Kollros and McMurray [11], and Weiss and Rossetti [22] have all reported the influence of thyroid hormone in inducing precocious growth and differentiation of cells of the larval frog brain. Nonetheless, not all brain cells show such hormone-induced precocity ([21]; Kollros and Barch, unpublished). While many brain cells undergo further maturation as a normal aspect of metamorphosis, the two giant Mauthner's cells (hereafter referred to as M-cells) involute during this period [18]. This regression of the M-cell is dependent upon thyroid hormone [2, 3, 22, 1]. Although Weiss and Rossetti interpret their results as examples of *direct* action of the hormone upon the M-cell, many of their tadpoles showed other marked metamorphic changes. It is possible, therefore, that the atrophy of the M-cells of their tadpoles might have been influenced indirectly through some other hormone-induced metamorphic event.

In the work of Weiss and Rossetti the differences in the sizes of non-M-cell nuclei between control and hormone-treated larvae were in each instance highly significant. In contrast, the size differences of the M-cell nuclei yielded a P value of only 0.02 in their *Xenopus* series and in one of the two *Rana* series it was barely significant ($P = 0.05$). In another *Rana* series the differences were not significant.

It seemed desirable, therefore, to reinvestigate the problem of M-cell involution, employing techniques permitting greater distinction between local and general effects of hormone treatment. This is possible with the use of thyroxine-cholesterol pellets [10]. By implanting the pellets along the side of the hindbrain, rather than in the fourth ventricle or on the choroid plexus,

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less hormone was expected to be lost to the general circulation than in the experiments of Weiss and Rossetti, and thereby less stimulation for general metamorphosis was anticipated.

MATERIALS AND METHODS

Rana pipiens larvae were obtained from eggs by Rugh's method [16] or collected from ponds. They were raised in aquaria with boiled lettuce constantly present. After operation they were kept in separate bowls.

The hormone pellets were prepared in a Hartman apparatus modified according to Kaltenbach [6], from a mixture of 20 per cent thyroxine (E. R. Squibb & Sons) and 80 per cent cholesterin (Merck, U.S.P.). They measured 0.5 mm by about 0.14 mm.

The staging referred to in this paper is that of Taylor and Kollros [19]. Larvae of stages varying from IV to XIII were anesthetized in 2.5 per cent ethyl urethane solution and transferred to Holtfreter's solution. In each, a pellet was implanted against the right side of the hindbrain at the level of entrance to the eighth nerve. Five to ten days later the larvae were fixed in Bouin's solution. The heads or brains were serially sectioned at 10 μ, and stained with Delafield's hematoxylin. The left sides of the hindbrains served as controls for the thyroxine-treated right sides.

In each animal the largest cross sections of the right and left M-cell nuclei (containing nucleoli) were drawn at a magnification of 1790 × with the aid of a camera lucida. Similarly, the nuclei of 30 "large" and 50 "small" non-M-cells from each side were drawn. To avoid measuring nuclei more than once, every third section in the vicinity of the M-cells was used.

The distinction between "large" and "small" cells was made as follows : The large cells were aggregated in a nucleus in the hindbrain mantle, mediodorsal to the M-cell (the nucleus motorius tegmenti, the motor nucleus of the seventh nerve, or a combination of both, [14, 15]). Only those large-cell nuclei containing distinct single nucleoli were selected. Small cells were selected from the area dorsolateral to the nucleus of large cells; their nuclei were reticulated and did not possess distinct single nucleoli.

The areas of all nuclear drawings were determined with the polar planimeter. Those of the M-cells were converted into units of μ^2 and the statistical significance of the differences between left and right M-nuclei was computed, the method of paired comparisons being used. The Chi Square test was utilized in the evaluation of differences in the distribution of nuclear sizes between the control and hormone-treated non-M-cells. Measurements for all animals were pooled and the test was performed on data for all large non-M-nuclei (A) and all small non-M-nuclei (B).

RESULTS

Nineteen animals were studied. All of them showed some degree of generalized developmental stimulation as a result of thyroxine released from the thyroxine-cholesterol pellet. Considered together, the M-cells on the side containing the pellet were insignificantly smaller than those on the opposite

TABLE I

Nuclear size of Mauthner's cells differentially exposed to thyroxine from a local unilateral source.

Case	Stage at oper.	Size in mm	Stage at fix.	Size in mm	Time in days	M-nucleus area in μ^2		Difference Left − Right
						Left	Right	
T 8	IV	29	VII+	26	6	194	175	19
T19	X	53	XIII−	58	5	223	248	−25
T20	IV	37	XI	50	10	224	243	−19
T23	VI	47	XVIII	57	10	203	247	−44
T24	VIII+	47	XIII	50	8	302	231	71
T30	X	55	XIV	60	7	279	220	59
A T32	X−	52	XIV	55	7	294	327	−33
T34	VI	42	X	51	7	218	217	1
T39	XII−	52	XVII+	57	7	200	190	10
T42	VI	42	XVIII−	50	8	171	201	−30
T50	IV+	34	XVII+	33	8	199	223	−24
T55	VI	43	XVII+	44	8	166	227	−61
T56	X	52	XVIII	52	8	204	153	51
Average	VII+	45	XIV+	49	8	221	223	−2
				$P=0.85$				
T45	V	42	XX	44	8	238	188	50
T47	V+	41	XXI	43	8	188	174	14
T51	V+	39	XXI	36	8	183	168	15
B T52	IV−	31	XX	28	8	193	131	62
T53	III+	33	XX	34	8	221	186	35
T54	VI	39	XX	39	8	154	138	16
Average	V−	38	XX+	38	8	196	164	32
				$P=0.02$				
Overall average	VII−	43	XVI+	46	8	213	205	9
				$P=0.33$				

side (Table I, $P=0.33$). If the 13 animals which were stimulated toward metamorphosis only slightly or moderately are considered separately (group *A* of Table I), they too have left and right M-cells of essentially identical sizes ($P=0.85$). In contrast, the six animals which were stimulated strongly, i.e., to the stage of forelimb emergence or beyond (group *B* of Table I), show a significant difference in the sizes of their two M-cells ($P=0.02$). In all six the M-cells on the right side, near the pellet, have smaller nuclei than those on the left side, more distant from the pellet. In comparing

tadpoles of groups *A* and *B*, it is obvious that the average initial stage of the former is more advanced than that of the latter, but that it progressed less. The average size also is greater in *A*, and during the experimental period only the animals in group *A* grew (average of 4 mm vs. 0 mm). These figures support the idea that generalized metamorphic stimulation was substantially greater in group *B* than in group *A*.

In Table II the 570 large non-M-nuclei and the 950 small non-M-nuclei on either side of the hindbrains are compared separately. In both the size distribution is very significantly different ($P = 0.001$), and the nuclei tend to be larger on the side of the brain containing the pellet. These results confirm those of Weiss and Rossetti. In all 19 animals the large non-M-nuclei were larger on the pellet side than they were contralaterally. The same was true of the small non-M-nuclei in all but two tadpoles. In most instances, however, statistical significance of differences in size of non-M-nuclei between the two sides of *individual* animals could not be established.

TABLE II

Nuclear size classes of two different groups of non-Mauthner cells differentially exposed to thyroxine from a local unilateral source.

	Nuclear area in planimeter units[1]					
A. Large non-M-nuclei						
Side	43–66	67–86	87–106	107–126	127–146	147–171
Left	129	284	119	33	6	0
Right	60	232	215	52	9	2
			$P < 0.001$			
B. Small non-M-nuclei						
Side	20–29	30–39	40–49	50–59	60–69	70–78
Left	25	296	474	135	17	3
Right	12	188	458	250	40	2
			$P < 0.001$			

[1] 1 planimeter unit = 1.1 μ^2.

DISCUSSION

Baffoni, Baffoni and Catte, and Weiss and Rossetti [1, 2, 3, 22] have reported that the atrophy of the M-cell which accompanies metamorphosis [18] may be precipitated or accelerated by stimulation of the entire animal with strong doses of thyroid hormone. This is also suggested in the present study (group *B* of Table I). In addition, the *local* influence of the

hormone on the M-cell is indicated by the size differences between cells of the right and left sides. Further, the absence of size differences between M-cells of the two sides in animals whose general metamorphosis was stimulated only moderately (group *A* of Table I), when at the same time both groups of non-M-cells developed such differences, shows clearly that the M-cells have a different threshold of response to thyroxine than the two classes of non-M-cells. It is not clear, however, whether the response of the M-cells is due solely to the local stimulation, since generalized metamorphosis was so prominent in the group *B* tadpoles. The influence of lateral line centers in the brain and of motor centers in the cord in maintaining the M-cells is recognized by Stefanelli [18]. Thus the influence of the prematurely degenerating lateral line centers in the brain [3] and of the degenerating motor system of the tail cannot be discounted despite the unilateral central response. It is possible that both a high local concentration of hormone and prior degeneration of lateral line and tail motor centers are required for M-cell involution. The failure of Weiss and Rossetti to obtain involution of the M-cell in non-metamorphosing tadpoles after tail amputation or unilateral hemisection of the spinal cord high in the trunk, is consistent with this possibility. In contrast, Stefanelli [17] reports involution of M-cells developed within explants of presumptive medulla, or in fragments of the presumptive Mauthnerian zone transplanted into the spinal cord. He attributes the involution to the lack of functional activity. He also concludes [18] that "the M-apparatus can exist only when both the sensory system of the lateral line and the motor system of the tail are present". In preparations of head grafts of *Rana pipiens* (Kollros, unpublished; but see Kollros, 1949), containing the Mauthnerian part of the medulla, M-cells develop almost normally, and do not degenerate until the metamorphosis of the host occurs, i.e., they remain intact far longer than the 20–30 days reported by Stefanelli. In these preparations tail and trunk motor cells are eliminated completely, and lateral line organs are sharply reduced in number, emphasizing the role which thyroid hormone may play in M-cell involution.

Similar experiments with local thyroxine-pellet stimulation of the specialized cells of the mesencephalic V nucleus and contrasting experiments of thyroid gland inhibition with thiourea [13, 8, 9, 12, 11] give remarkably uniform results. These results indicate that in the larval period the level of differentiation attained by the cells of the mesencephalic V nucleus is controlled by the amount of thyroid hormone available, and that some degree of this control continues postmetamorphically. Although these cells have higher thyroxine thresholds than do other metamorphic events up to and

including forelimb emergence, it is quite obvious that they have lower thresholds than have the involuting M-cells.

The present study supports the findings of Weiss and Rossetti that the hindbrain contains neuron types qualitatively different with respect to their capacity to react to thyroid hormone. It shows, further, a substantial difference in thresholds for the reaction of the various cell types. However, a decision as to whether thyroxine acts only directly, or indirectly as well, in producing atrophy of the Mauthner's cells must await further investigations.

SUMMARY

1. Thyroxine-cholesterol pellets were implanted against the right side of the hindbrain in *R. pipiens* larvae.

2. Nuclei of two different classes of non-Mauthnerian cells on the side of implantation grew significantly larger than did the same cell types on the opposite (control) side. In contrast, the sizes of the Mauthner cell nuclei showed no significant differences between the two sides, except perhaps in a selected small group in which much of the hormone must have gone into the circulation, since marked generalized metamorphic advances occurred.

3. It is concluded that involution of Mauthner's cells requires either high concentrations of thyroid hormone locally for several days, or high concentrations of the hormone locally in addition to prior degeneration of lateral line nerve centers or tail motor centers.

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BRIEF NOTES

THE CYTOPLASMIC DISTRIBUTION OF *ISOCITRIC* DEHYDROGENASES

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There are two enzymes known to catalyze the oxidation of *iso*citrate, one having triphosphopyridine nucleotide (TPN) and the other diphosphopyridine nucleotide (DPN) as specific coenzymes. The cytoplasmic distribution of the TPN-linked enzyme has been investigated by Hogeboom and Schneider [7], who found that over 80 per cent of the activity present in liver homogenates is recovered in the soluble fraction, while only about 12 per cent is located in the mitochondria. It has been pointed out by these authors [10, 6, 9] that the relatively low activity found in mitochondria might be due to non-mitochondrial contamination. The DPN-linked *iso*citric dehydrogenase has been purified by Plaut and Sung [8] from water extracts of acetone-treated beef and guinea-pig heart mitochondria, and found to possess an activity about thirty times lower than the TPN-linked enzyme present in the same extracts. The cytoplasmic distribution of the DPN-linked enzyme has not been investigated in these studies.

In the present paper it is shown that in rat liver cytoplasm the DPN-linked *iso*citric dehydrogenase is located exclusively in the mitochondria. Its activity is about one-fortieth of that of the total cytoplasmic TPN-linked *iso*citric dehydrogenase. The cytoplasmic distribution of the latter has been found to be in agreement with the data of Hogeboom and Schneider [7]. The evidence obtained indicates that the fragment of the TPN-linked *iso*citric dehydrogenase found in the mitochondrial fraction is truly mitochondrial, and not due to contamination, but of the two kinds of *iso*citric dehydrogenase present in mitochondria mainly the DPN-specific enzyme is involved in the aerobic oxidation of *iso*citrate.

The determination of the *iso*citric dehydrogenase activities was performed by following the reduction of added DPN or TPN in a Beckman DU spectrophotometer at 340 mμ. The extinction was recorded at one minute intervals for five minutes. One-cm glass cuvettes were employed, the light path being reduced to 1 mm by plungers. Each cuvette contained 1.3 ml of a solution containing 0.25 *M* sucrose, 0.02 *M* potassium phosphate buffer (pH 7.2), 9.2×10^{-4} *M* $MnCl_2$, and, when the mitochondrial fraction was to be tested, 7.7×10^{-4} *M* KCN in order to suppress respiration. To this, 0.1 ml 0.03 *M* DPN or TPN was added, followed by 0.2 ml of

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TABLE I

Cytoplasmic distribution of *iso*citric dehydrogenases.

Values referred to 100 mg liver. Number of experiments in parenthesis.

Fraction	DPN-linked		TPN-linked	
	Micromoles DPNH per 5 min.	Per cent distribution	Micromoles TPNH per 5 min.	Per cent distribution
Mitochondrial*	0.43±0.28 (11)	100	1.69±0.43 (15)	10
Soluble . . .	0.00 (24)	0	14.75±2.36 (44)	90

* Aged for 60 min. (cf. Fig. 1).

TABLE II

Influence of manganese and adenosine triphosphate on mitochondrial TPN-linked *iso*citric dehydrogenase activity.

Values referred to 100 mg liver.

Expt. No.	System	Micromoles TPNH per 5 min.
1	fresh	0.58
	aged 30 min.	2.65
	aged 30 min. in presence of 0.5 m*M* Mn^{++}	1.45
	aged 60 min.	3.19
	aged 60 min. in presence of 0.5 m*M* Mn^{++}	2.17
2	fresh	0.48
	aged 45 min.	2.07
	aged 45 min.; 2.5 m*M* ATP added to test incubation	0.51

the enzyme to be tested. The reaction was initiated by the addition of 0.2 ml 0.1 *M* potassium DL-*iso*citrate. The blank contained all additions except *iso*citrate, which was replaced by 0.2 ml sucrose-phosphate buffer.

Twice washed rat liver mitochondria were prepared in 0.25 *M* sucrose, according to Schneider and Hogeboom [11]; the detailed procedure has been described elsewhere [4]. The supernate obtained was centrifuged at 60,000 *g* in a Model L Spinco Ultracentrifuge, Rotor No. 40, for 30 minutes at 0°C. The microsomal fraction, which was found to be devoid of *iso*citric dehydrogenase activity, was discarded. The soluble fraction from one liver was made up to 100 ml with 0.25 *M* sucrose and tested, either

directly, or after dialysis overnight against deionized water at 0°C, after which the enzymic activity was found to be unaltered.

Mitochondria from one liver were suspended in 30 ml of a solution containing 0.083 *M* sucrose, 0.1 *M* KCl, 0.033 *M* potassium phosphate (pH 7.2), 2.85×10^{-3} *M* potassium adenosine-5-phosphate, 0.04 *M* glucose, 0.002 *M* $MgCl_2$, and an excess of yeast hexokinase. In order to obtain maximum rates of DPNH or TPNH formation, the mitochondria were subjected to aging at 30°C. The effect of aging is illustrated

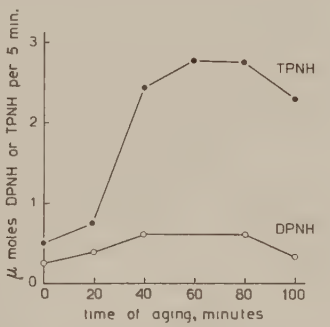

Fig. 1. Effect of aging on the apparent *iso*citric dehydrogenase activity in rat liver mitochondria. Values referred to 100 mg liver.

by Fig. 1. Maximum rates appear after about 60 minutes; upon prolonged aging the rates tend to decrease. Oxygen uptake was measured in conventional Warburg manometers, using 1.5 ml aliquots of the mitochondrial suspensions, supplemented with 0.1 ml 0.2 *M* potassium DL-*iso*citrate, 0.1 ml 0.01 *M* $MnCl_2$, 0.1 ml 0.05 *M* potassium adenosine triphosphate, 0.1 ml 6×10^{-4} *M* cytochrome *c*, and, where indicated, 0.1 ml 0.015 *M* DPN or TPN. All measurements were performed at 25°C.

The cytoplasmic distribution of the DPN- and TPN-linked *iso*citric dehydrogenases is shown in Table I. As seen, the DPN-linked enzyme is absent from the soluble frac-

TABLE III

Effect of added pyridine nucleotides on the aerobic oxidation of *iso*citrate in aged mitochondrial systems.

Values referred to 100 mg liver.

Aging time, minutes	Oxygen consumption, microatoms per 5 min.		
	No pyridine nucleotide added	DPN added	TPN added
0	0.35		
30	0.13	0.31	0.19
60	0.05	0.30	0.08

tion. The distribution of the TPN-linked enzyme between soluble fraction and mitochondria is 9:1. The total activity of the TPN-linked enzyme exceeds that of the DPN-linked one by a factor of about 40. The TPN-linked enzyme found in the mitochondrial fraction appears to be specificly mitochondrial, since it does not exhibit maximum activity unless the mitochondrial structure is altered by aging (Fig. 1). Moreover, as shown in Table II, the effect of aging can be delayed by manganous ions (Expt. 1), or reversed by adenosine triphosphate (Expt. 2). These properties have previously been found to be typical for true intramitochondrial dehydrogenases [2, 3, 5].

Although the activity of the mitochondrial TPN-linked enzyme is, in terms of *iso*citrate oxidation, about four times higher than that of the DPN-linked enzyme (Table I), the latter seems to be the almost exclusive pathway of *iso*citrate oxidation under aerobic conditions. Thus, as shown in Table III, if the mitochondria are depleted of their complement of pyridine nucleotides by aging [2, 3], addition of DPN, but not of TPN, restores the aerobic oxidation of *iso*citrate. The failure of TPN to restore respiration may be due to the relatively poor TPNH-cytochrome *c* reductase activity present in mitochondria [1].

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BIOCHEMICAL STUDIES ON THE INDUCTION OF SYNCHRONIZED CELL DIVISION

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A short period of chilling applied to actively multiplying bacteria results in synchronous cell division for several subsequent generations [3, 4, 10]. This inductive

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effect of cold was postulated to be based on the differential effect of cold on particular synthetic activities in bacteria [4]. One of the hypotheses assumed that the rate of desoxyribonucleic acid (DNA) synthesis may be selectively reduced at lower temperatures [7], while cytological studies indicated an increased proportion of chromatinic material in chilled *Bacillus megaterium* [5, 10]. In the present studies, an attempt was made to measure the progress of the synthesis of DNA and of other cell compo-

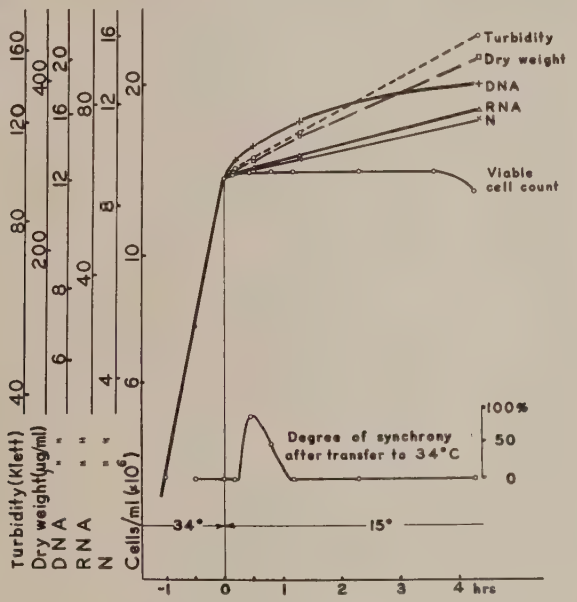

Fig. 1. Differential effect of chilling on cell division and on synthetic activities of *Bacillus megaterium*.

nents during the growth of bacteria at optimal temperature followed by incubation at low temperature (15°C). In addition the inducing effect of various periods of chilling on the initiation of synchronous cell division was tested.

Methods.—The methods employed for induction of synchronous multiplication of *B. megaterium* were described previously [5]. The liquid medium contained 1 per cent casein hydrolyzate and a buffered mixture of salts (7 g K_2HPO_4; 2 g KH_2PO_4; 0.1 g $MgSO_4 \cdot 7H_2O$; 0.5 g sodium citrate per liter). The use of large volumes of broth (1–2 liters) was imperative because on the one hand, the titer of the cells had to be relatively low, in the neighborhood of 10^7/ml, for successful induction of synchronous multiplication, and on the other hand, the need for numerous samples required a considerable initial volume of liquid. Samples were collected for three types of assays: viable count, degree of induction of synchrony, and biochemical analyses. Viable counts were determined by spreading an appropriate dilution on the surface of a pre-dried agar medium. Pouring methods were not successful because of excessive clumping of the cells. The degree of synchronization was measured by transferring

the samples to optimal temperature (34°C) and assaying the number of cells every 5 minutes for a period of 1 ½–2 hours as previously described [5]. Biochemical analyses included the determination of DNA, ribonucleic acid (RNA), and protein, together with total dry weight and turbidity assay. Analytical methods were carefully standardized so as to assure their reproducibility in serial assays designed principally to measure the relative changes in cell components rather than their absolute content. For DNA, RNA, and protein analyses, 8 ml samples were mixed with 2 ml of prechilled 50 per cent perchloric acid (PCA). Cells were extracted for 30 minutes at 0°C, and washed twice with cold 10 per cent PCA. Hot treatment (15 minutes at 90°C) and washing with 5 per cent PCA were used for quantitative separation of the nucleic acid fraction, which was analyzed colorimetrically for DNA [9] and RNA [8]. Parallel determinations by ultra-violet absorption (260 mμ) of nucleic acids in the hot PCA extract were in very good agreement ($\pm$ 5 per cent) with the colorimetric assays. The residue was mineralized (heated with conc. H_2SO_4 and H_2O_2 as catalyst), and used for nitrogen (protein) determination by direct Nesslerization [6].

For dry weight determination an 8 ml aliquot was centrifuged, washed, and dried to constant weight at 100°C.

Bacterial growth at 34°C was followed by standardized turbidity determinations (Klett, blue filter). Samples for analytical determination were taken at turbidity levels corresponding to approximately 5×10^6 cells/ml followed by two more samples taken during the logarithmic growth period. Chilling coincided with the third sampling, and was executed by transferring the whole flask from a 34° bath to another water-bath shaker set at 15°C [5].[1] The second series of samples was taken at ½ hour, 1 hour and 3 hours after chilling.

Results and Discussion.—Fig. 1 illustrates the course of syntheses before and after chilling. The curves, which represent the averages from 6 experiments, were plotted so as to meet at the same point, corresponding to the time of chilling. The rates of all the synthetic processes were identical during logarithmic growth and are represented by a straight line on the semilogarithmic plot. The subsequent decrease in the temperature of incubation had differential effects on the measured processes as illustrated in the diagram (Fig. 1). Cell multiplication was almost completely arrested within 30 minutes after chilling. Turbidity of the culture, on the other hand, seemed to be least affected. Changes in dry weight closely followed the turbidity curve. The fact that progress of DNA synthesis was less inhibited than the protein synthesis, explains the increase in stainability of nuclei in thionin-stained cytological preparations (compare Figs. 1 and 2; [5]). Similarly, the observed increase in cell length (see Figs. 4–8; [5]) is an obvious result of the disproportion between cell division and synthesis of cellular materials (Fig. 1). Measurements of the degree of synchronization indicated that the highest degree of simultaneous multiplication (75–95 per cent of the cells) could be attained when the chilling period (15°C) was around 30 minutes.

[1] In order to speed chilling in a few exploratory experiments, the flask was first immersed for two minutes in ice water. This procedure, however, was accompanied by a 10–20 per cent drop in the viable count, together with similar decreases in the DNA, RNA, and protein content of the cells. Only the turbidity curve remained undisturbed. A lethal effect of rapid chilling, together with increased fragility of the cells, was probable; partial lysis during subsequent handling apparently resulted in a partial loss of the components to be analyzed. This lethal effect of cold on actively multiplying bacteria was previously reported [3] and ascribed to its effect on the electrolyte balance [11].

It is difficult to relate this very short period to any particular event in the synthesis of those cell components which are represented by the curves in Fig. 1. It may not be merely coincidental, however, that the period of highest induction of synchrony corresponds to the period of highest discrepancy between the rates of DNA and protein synthesis and to the interval immediately following the sharp arrest of cell multiplication. The more immediate effect of chilling on protein synthesis, as compared with lesser effect on DNA synthesis, may indicate that the pool of DNA intermediates is larger than the corresponding protein-precursor pool. Further systematic studies of the influence of temperature changes on the rates of synthesis of DNA, RNA, protein, their building blocks, and high energy intermediates might lead to a better recognition of the reactions primarily responsible for the arrest of cell division. This almost complete arrest cannot be accounted for by a comparable decrease in any of the measured synthetic processes. The synchronization process may depend on a limited period of unbalanced growth when preferential inhibition of the synthesis of one of the components brings the majority of the cells, or their nuclei, "into phase".

The possibility exists that differential conditions may produce the same result via different mechanisms. Thus, in the present case of synchronization of division through chilling, protein synthesis appeared to be most affected; in contrast, synchronization achieved by thionine deprivation in a thymine-requiring strain of *E. coli* appeared to involve a primary effect on DNA synthesis [1]. No synchronization was achieved in *E. coli* by simultaneous blocking of DNA and protein synthesis [2].

Summary.—Thirty to sixty minutes chilling (15°C) of actively multiplying cultures of *B. megaterium* induces a synchronous cell multiplication on subsequent incubation at 34°C. Cell division is completely arrested within 30 minutes of the chilling period. This effect could not be correlated with a comparable arrest of synthesis of any of the measured cell components, all of which are affected to a lesser degree. Dry weight and turbidity are influenced least by the decrease in temperature. Syntheses of DNA, RNA, and protein are affected in increasing order. These results are in agreement with semi-quantitative cytological observations indicating an increase in cell volume and in proportion of nuclear material in cold-arrested cells of *B. megaterium*. Synchronization seems to be the product of a critical period of "unbalanced growth", which by favoring DNA synthesis may permit all the nuclei to be brought "into phase" in the non-multiplying cells. This conclusion is necessitated by the inability to associate, so far, the complete arrest of nuclear and cellular division with a corresponding drastic alteration of any specific reaction.

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SUR L'ESPACE PÉRINUCLÉAIRE

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Par microcinématographie en contraste de phase, sur des leucocytes soumis à des milieux hypotoniques ou en voie d'autolyse débutante, on peut voir se former, entre le noyau et le cytoplasma, des vacuoles en croissant, à contenu clair au contraste de phase. Elles s'étendent progressivement sur une surface de plus en plus grande du noyau, en se fusionnant quelquefois.

De telles vacuoles internucléocytoplasmiques avaient été signalées par Dustin [4] dans l'ordre histologique et étudiées par nous-mêmes en microcinématographie en 1953 (Fig. 1).

Au microscope électronique, sur des coupes ultraminces faites suivant la technique habituelle, on peut préciser la situation exacte de ces vacuoles. Elles résultent de l'agrandissement de l'espace clair non osmiophile, de 400 à 800 Å environ qui sépare les deux minces membranes osmiophiles (40 Å environ) marquant les limites du cytoplasma et du noyau. Sur ces coupes, on peut voir nettement la zone de contact nucléocytoplasmique, zone en rapport souvent avec les « pores » et les « anneaux » nucléaires signalés dans diverses cellules et de signification encore mal précisée (cf. [3, 5, 1, 8]).

Ces constatations inframicroscopiques permettent d'envisager l'existence, entre noyau et cytoplasma, d'un zone périnucléaire spéciale capable de gonflement et de décollement. Dans cet espace peuvent pénétrer, le cas échéant, diverses formations cellulaires extérieures. Un exemple typique est celui des voiles cytoplasmiques vus en microcinématographie par Robineaux [9] au cours de la phagocytose du noyau altéré des cellules de Hargrave. Ces voiles se glissent entre cellule et noyau en suivant l'espace périnucléaire. Peut-être aussi pourrait-on penser aux filaments nerveux signalés par Weber [11] entre noyau et cytoplasma.

La notion d'un espace périnucléaire gonflable et décollable peut aussi être rapprochée des faits intéressants signalés récemment par Lettré et Schleich [6] sur le mécanisme de l'expulsion du noyau, vue au microcinéma, au niveau d'érythroblastes et de diverses cellules cultivées *in vitro*. Sous l'influence d'un ATP purifié, il y a séparation du noyau et du cytoplasma au niveau de l'espace périnucléaire, contraction du cytoplasma en quelques masses et expulsion du noyau.

La structure inframicroscopique de l'espace périnucléaire reste à préciser. Toutefois, à son sujet, quelques faits peuvent être évoqués. Par utilisation de techniques très perfectionnées de polarisation, Baud [2] a pu montrer que la zone périnucléaire dans son ensemble paraît constituée de molécules protéiques dans un état intermédiaire entre molécules globulaires et filamenteuses. Sa biréfringence étant très faible à l'état normal, on doit admettre que les molécules sont dans un état incomplè-

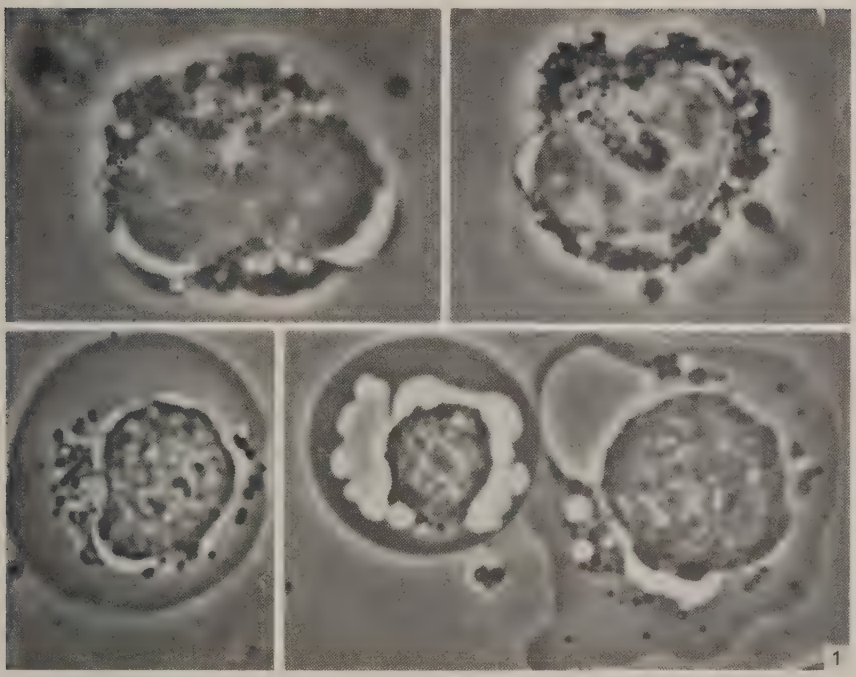

Fig. 1. Vacuoles périnucléaires en croissant à différents stades d'évolution. Contraste de phase. Images extraites d'un film.

tement orienté et fortement hydraté. Au microscope électronique, aux plus hautes résolutions possibles pour nous, le contenu de cet espace périnucléaire nous est toujours apparu homogène et clair. Les deux membranes n'ont pas encore montré de signes nets d'orientation.

Certaines images microélectroniques permettent de considérer comme possible l'ouverture, dans l'espace périnucléaire, d'éléments vacuolaires ou canaliculaires du réticulum endoplasmique de Porter Palade et de confirmer ainsi les constatations de Watson [10] sur la continuité directe entre « cisternae » cytoplasmiques et enveloppe nucléaire. Il existerait, à ce niveau, dans la cellule, les mêmes communications signalées, on le sait, à la surface extérieure de la cellule. Nous avons constaté, en particulier, qu'un gonflement de l'espace périnucléaire apparaissait toujours après action d'une hypotonie du milieu. Ces faits ouvrent des vues nouvelles et importantes sur les mécanismes cellulaires.

La zone périnucléaire nous apparaît constituer une région très spéciale de la cellule. Son comportement ne se ramène pas à celui d'une simple membrane limitante ou d'une interface.

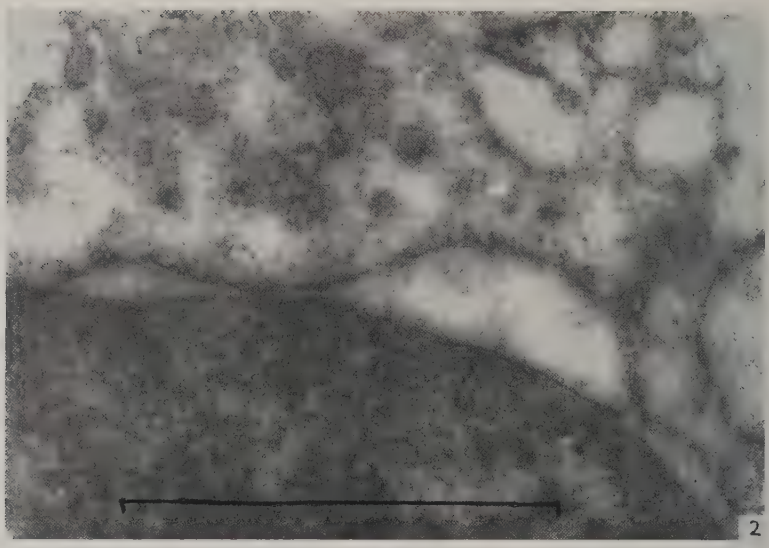

Fig. 2. Début de la formation de vacuoles périnucléaires (microscope électronique). Leucocyte. Fixation Palade.

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THE ULTRASTRUCTURE OF SKELETAL MUSCLE MYOFILAMENTS AT VARIOUS CONDITIONS OF SHORTENING

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In the present work special attention has been paid to the study of longitudinal sections through myofibrils. Pictures of very thin sections have been taken at an electron optical magnification of 40,000 to over 60,000 times. A well compensated double objective pole piece without any aperture has been used in an RCA EMU 2 *c* electron microscope. The use of an aperture could be avoided by mounting the sections on formvar nets [7, 8] instead of films. The sections were cut on Sjöstrand ultra-microtomes [6] manufactured by LKB Products.

Muscles of mouse and frog have been fixed in buffered osmium tetroxide solution at or below 0°C. Phosphotungstic acid stain has been applied during the dehydration in alcohol. Contraction of the muscle has been produced through direct electric stimulation during the fixation.

Results.—According to the most common observations there exists only one kind of myofilaments which extend without interruption through the whole sarcomere. This fact gives no support for the hypothesis dealing with the morphology of muscular contraction presented by Hanson and Huxley [1, 2] but is in agreement with the observations made on dipteran flight muscles [3, 5].

Both the diameter and the inner structure of the individual myofilaments vary in the different cross bands of the sarcomere. Within the *I* band the myofilaments measure about 40 Å in thickness and show a rather smooth cylindrical shape. The *A* bands are characterized by the greater and more variable thickness of the myofilaments (Fig. 1). The contours of the myofilaments within the *A* band are very irregular due to alternating constrictions and bulges and may appear similar to the edge of a saw blade. At the thicker regions adjacent myofilaments frequently are interconnected through transversally orientated 20–30 Å thick bridges. The distances between such cross connections vary with the degree of shortening of the sarcomere between about 250 and 90 Å. The thickness of the thinner regions of the myofilament in the *A* band also varies with the degree of shortening of the sarcomere between 60 and 140 Å.

The opacity of the myofilaments varies along their length in an irregular fashion and in shortened sarcomeres an irregular cross banding is indicated. This cross banding is interpreted as showing the existence of structural subunits in the myofilament which are predominantly transversally oriented in the shortened sarcomeres.

The appearance of the myofilaments in the *A* band at different degrees of shortening may be interpreted as due to a coiling of smaller filamentous subunits with the pitch of the helix varying with the degree of shortening. The thickness of these sub-

Fig. 1. Longitudinal section through the *A* band of frog skeletal muscle. In the upper right corner part of one *I* band. The diffuseness of the myofilament structure is due to diffuse electron staining and not to optical unsharpness. Shortened sarcomere. Magnification × 230,000.

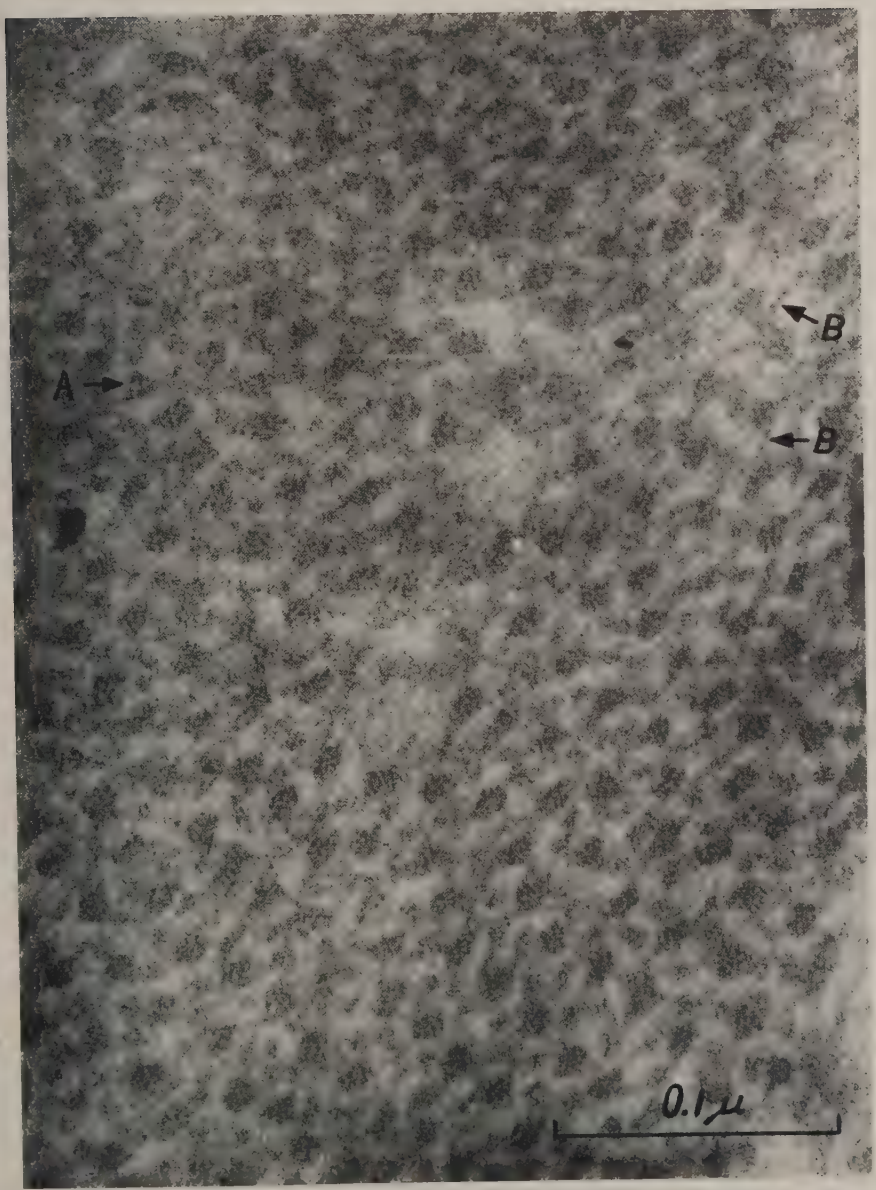

Fig. 2. Cross section through the *A* band region of frog skeletal muscle. Notice the angularly shaped cross section of the myofilaments. At *A* a triangular cross section and at *B* interconnecting bridges about 20 Å thick. The granularity is due to phase contrast effects depending on a slight degree of underfocussing. This superimposed granularity might mask a possible granular structure of the myofilaments. Magnification × 420,000.

units is preliminarily estimated to about 30 Å. Other interpretations are, however, possible and different alternatives are being considered as this study proceeds.

The myofilaments with interconnecting bridges form a three dimensional network which, however, is far less regularly arranged in mammalian skeletal muscle than in the dipteran flight muscle studied by Hodge [3].

In cross sections (Fig. 2) a considerable number of myofilaments show triangular outlines with the interconnecting bridges extending between the corners of the triangles.

At the *M* bands the myofilaments appear smoother and slightly thicker with rather broad diffuse transversal connections between adjacent myofilaments. On muscle tissue fixed in the fresh state without any preceding extraction procedure no noticable difference in the appearance of the myofilaments has been observed within the *H* band as compared to the rest of the *A* band.

Shortening of the muscle fibers produced through direct electric stimulation is associated with a reduction of the height of the *I* bands as well as the *A* bands, which confirms the results of Knappeis and Carlsen [4]. As no new structural pattern appears in connection with the reduction of the *I* band the conclusion may be drawn that the *I* band pattern has partially changed into an *A* band pattern. Intermediate stages between these two pattern may be observed.

A consequence of such an interpretation of the observations made will be that the cross striated muscle myofilament in the extended condition consists of alternating "coiled" (*A* band) and "extended" (*I* band) regions. The *I* band may represent a specialized region adapted to increase the range of shortening through a transfer from an "extended" to a "coiled" state. A decoiling of the *A* band region ("plasticizing") might extend the range of lengthening of the myofilament.

Even if the interpretation presented here might seem consistent with the observations made so far during this study, we have to stress its presumptive character. The structural organization of the myofilaments is a complicated matter when studied at this high resolution. The amount of information we may obtain is for the time being restricted by the diffuseness of the electron staining of the subunits in the myofilaments. There is no justification as yet to become fixed to any simplified model conception regarding the morphology of muscle contraction.

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EFFECTS OF PROTEOLYTIC ENZYMES ON THE ZONA PELLUCIDA OF FERTILIZED AND UNFERTILIZED MAMMALIAN EGGS

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According to the observations of Braden, Austin and David [2] the penetrability of zona pellucida to sperm is not influenced by the entry of the first sperm in the rabbit egg. Thus, 62 sperm could be found in the perivitelline space of an egg. Penetrability decreases after the entry of the first sperm in the rat and the mouse egg, for the number of eggs that contain more than one sperm was much smaller than would be expected. The zona pellucida of sheep, dog and probably hamster [3] eggs, however, precludes further penetration of sperm after the entry of the first one because no extra sperm were detected in the perivitelline space of a fertilized egg. Evidence of a change in the zona pellucida has been reported by Smithberg [4] who observed that the zona pellucida of unfertilized mouse eggs was invariably removed by some proteolytic enzymes before that of fertilized eggs. This preliminary study was planned to determine whether the effects of proteolytic enzymes on the zona pellucida differs in the fertilized and unfertilized eggs of rabbit, rat and hamster, and whether the difference in penetrability of zona to sperm could be demonstrated by enzyme reaction in the egg of these species.

The fertilized or unfertilized eggs were recovered by flushing the Fallopian tubes of rabbits 17–20 hours after mating or after an intravenous injection of gonadotropic hormone. They were obtained by dissecting the tubes of rats and hamsters about 12–19 hours after mating or after the observation of estrous behavior. Most of the fertilized eggs were at the pronuclear stage and the presence of sperm tail or intact sperm in the egg could be easily seen under a compound microscope. The eggs were first treated with hyaluronidase in order to dissolve the cumulus clot, but the corona radiata of rabbit eggs could not be dissolved by hyaluronidase. A few fertilized and unfertilized eggs were mounted on a slide under a coverslip supported by vaseline spots. Various proteolytic enzymes at a concentration of 0.1 per cent for the rabbit and rat eggs or at a concentration of 0.01 per cent for the hamster eggs in Ringer phosphate solution at pH 6.6 to 7.9, according to the specific activity of the enzymes tested, was applied between the slide and the coverslip. The edges of the coverslip were then sealed with vaseline. The eggs were examined at room temperature under a phase contrast microscope at various intervals.

Table I summarizes the results. It indicates that under the conditions of the experiments: (*a*) neither papain, fibrinolysis, nor hyaluronidase were able to effect zona digestion of any type of egg; (*b*) ficin, chymotrypsin, proteinase, pancreatin and lipase were ineffective in fertilized rat and rabbit eggs and relatively inactive in unfertilized eggs of these species; (*c*) trypsin was the most active digestive enzyme

TABLE I

Digestion of the zona pellucida of fertilized and unfertilized mammalian eggs.

Enzymes	Rabbit		Rat		Hamster	
	Fert.	Unfert.	Fert.	Unfert.	Fert.	Unfert.
Papain[1]	–	–	–	–	–	–
Ficin[1]	–	–	–	+?	++	++
Trypsin[1]	–	+	+	++	+++	+++
Chymotrypsin[2] . .	–	–	–	+?	++	++
Proteinase[1]	–	+?	–	+?	++	++
Pancreatin[1]	–	+?	–	+?	++	++
Lipase[1]	–	+?	–	+?	++	++
Fibrinolysin[3] . . .	–	–	–	–	–	–
Hyaluronidase[4] . .	–	–	–	–	–	–

[1] Products of Nutritional and Biological Corporation, Cleveland, Ohio.
[2] Products of Plant Research Laboratory, Bloomfield, N. J.
[3] Products of Parke, Davis & Co.
[4] Kindly supplied by the Wyeth Biological Institute.

of zona in all species; and (*d*) the hamster zona is more susceptible to digestion by a variety of enzymes than the zona of the other species' eggs.

The thickness of the zona pellucida of a hamster egg is very similar to that of a rat egg. It takes only 2–10 minutes in a solution of 0.01 per cent trypsin to dissolve the zona of a hamster egg whereas it takes about 20–120 minutes in a solution of 0.1 per cent trypsin to dissolve the zona of a rat egg. The difference of protein constituents of the zona pellucida in these two species is well demonstrated. The zona pellucida of a rabbit egg is much thicker than that of the rat or hamster egg and it takes about 30–180 minutes in a solution of 0.1 per cent trypsin to dissolve it. As the digestion of the zona pellucida is very fast in the hamster eggs, no obvious difference in time was observed between the fertilized or unfertilized hamster eggs, indicating that there is no striking difference so far as enzyme reaction is concerned in the hamster zona pellucida before or after sperm penetration. In the rat egg, the digestion of the zona pellucida by trypsin is sooner in the unfertilized eggs than in the fertilized eggs (Fig. 1). The time required to digest the zona pellucida of rat eggs by other proteolytic enzymes is not so clear-cut but the result of several tests indicate that the digestion of the zona occurs sooner or more frequently in unfertilized than in fertilized rat eggs. This suggests that there may be a change in the zona of the rat egg after sperm penetration. In the rabbit egg, probably due to the slow reaction of the proteolytic enzymes or due to the presence of corona radiata or of albumin coat in some cases, the digestion of the zona by enzymes is not clear-cut. Nevertheless, there are indications showing that the digestion of the zona by trypsin in the unfertilized rabbit eggs probably occurs sooner or more frequently than in the fertilized egg. This suggests a change in the rabbit zona either due to the presence of the

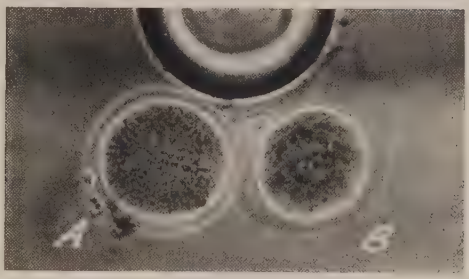

Fig. 1. *A*, a fertilized rat egg with intact zona pellucida; *B*, an unfertilized rat egg with zona pellucida partially dissolved, 20 min. after treatment with 0.1 per cent trypsin.

corona radiata or of the albumin coat as a result of time lapse after ovulation or due to the effect of sperm penetration.

During this study, the presence of several sperm in the perivitelline space of a rabbit egg was usual. The range was from 1 to 12 in 51 fertilized rabbit eggs examined. This count is not exact because more sperm may be present on the top or on the bottom of the perivitelline space which cannot be observed. Only a small portion of fertilized rat eggs contain more than one sperm in a total of 50 rat eggs examined, 3 had 3 sperm and 9 had two sperm. This count is quite accurate because the tail of the fertilizing sperm or the extra sperm can be seen easily by focusing the microscope. However, there was no fertilized hamster egg containing more than one sperm in a total of 64 fertilized eggs examined. It seems therefore that the blockage reaction of the zona pellucida in the hamster eggs after the entry of the first sperm is definite. The results, however, do not show that the zona pellucida of hamster eggs is more resistant to digestion after the penetration of the first sperm. The blockage of the zona in the rat and rabbit egg is not so striking and yet there are indications of differential enzyme reaction after sperm penetration.

It seems likely that there is a change in the protein constituents of the zona pellucida at various times after ovulation or after the penetration of sperm, which can be demonstrated by enzyme reactions in the rabbit and rat eggs. In the hamster eggs, although the blockage reaction of zona to further entry of sperm is more acute, yet this reaction cannot be demonstrated by alteration in proteolytic enzyme action under the conditions of these tests. A rather subtle change in the mucoprotein [1] of the zona pellucida in the eggs of these species may be significant to sperm penetration.

This study was supported by a grant from The Population Council. Thanks are due to Dr. G. Pincus for reading the manuscript.

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EIN BEITRAG ZUR ANALYSE DER WIRKUNG VON ÖSTRADIOL AUF DIE FRÜHE ENTWICKLUNG DES SEEIGELEIES

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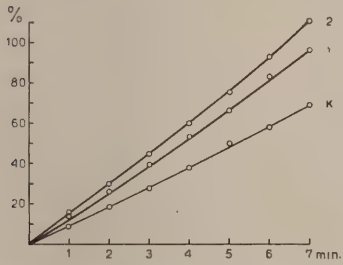
Es is mehrfach nachgewiesen worden, dass Östrogene als Mitosegifte wirken können [5]. Agrell [1] wies diese Wirkung bei den Eiern des Seeigels *Psammechinus miliaris* nach, die einer Behandlung mit Östradiol unterworfen wurden. An demselben Material zeigten Runnström und Kriszat [7], dass es in der frühen Entwicklung (etwa 1 3 Stunden nach der Befruchtung) ein für die Östradiolwirkung besonders empfindliches Stadium gibt und weiter, dass insbesondere die vegetativen Keimteile durch dieses Östrogen geschädigt werden. Angesichts dieser vom entwicklungsphysiologischen Standpunkt aus interessanten Tatsachen wird unten über Versuche berichtet, welche die Wirkung von Östradiol auf die Rigidität der Plasmamembran und des Endoplasmas mittels Zentrifugierungsversuche untersuchen. Ausserdem wird eine frühere Angabe [7] zahlenmässig belegt, nach der Adenosintriphosphat (ATP) der durch Östradiol verursachten Schädigung entgegenwirkt.

Material und Methode. — Alle Versuche wurden während des Sommers 1955 an der Zoologischen Station Kristineberg an der schwedischen Westküste durchgeführt. Die Eier von *Psammechinus miliaris* wurden in einem Seewasser von 25 pro mille gehalten, das ungefähr dem normalen Medium der litoralen Form dieser Art entspricht. Nach Behandlung mit Östradiol wurden die Eier anschliessend in eine Mikroküvette übertragen, wo sie zwischen Seewasser und einer isotonischen Zuckerlösung suspendiert wurden. Die in der Küvette suspendierten Eier wurden in einem Zentrifugenmikroskop nach Harvey und Loomis [2] für die Dauer von 7 Minuten mit einer konstanten Zentrifugalkraft von 4350 *g* zentrifugiert, vgl. [3]. Dabei wurde die Streckung der Eier gemessen, vgl. Fig. 1. Erfahrungsgemäss ist der Rigiditätszustand der Plasmamembran der wesentliche begrenzende Faktor bei der Streckung der Eier in einem Zentrifugalfeld. Beobachtungen über das Verhalten der Eier in einer hypertonischen Lösung [8] zeigten immer Ergebnisse, die mit den aus Streckungsversuchen gewonnenen gut übereinstimmten, vgl. [4]. Es muss dahingestellt bleiben, ob die „Plasmamembran" der ganzen Kortikalschicht des Eies oder nur dem äusseren Teil desselben entspricht.

Um die Rigidität des Endoplasmas zu bestimmen, wurde das gleiche Material unter Anwendung einer konstanten Zentrifugalkraft von 4460 *g* in einer gewöhnlichen Zentrifuge zentrifugiert, wobei die Eier zwischen Seewasser und einer reinen ca. 20%igen Gummiarabikum-Lösung von pH 8,2 suspendiert waren. Dabei unterliegen die Eier keiner Streckung. Die Eier, die eine deutliche Schichtung aufwiesen, konnten so leicht gezählt und mit einer gleichzeitig zentrifugierten, unbehandelten Kontrolle verglichen werden. Ausserdem wurden die unbefruchteten Eier nach entsprechender

Östradiol-Behandlung von 60 Minuten gleichzeitig mit einer unbehandelten Kontrolle in hypertonische Kochsalzlösung überführt (0.6 ml einer 2.5 *N* NaCl Lösung + 2 ml Seewasser), um die dabei auftretende Plasmolyse und deren zeitlichen Verlauf festzustellen. Für die ATP-Versuche wurde das kristallisierte Dinatrium ATP Sigmas benutzt.



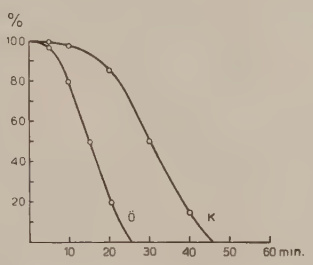


Abb. 1 (*links*). Verlängerung der Eier im Zentrifugenmikroskop nach Behandlung mit Östradiol. *K* = Kontrolle; **1** = Östradiol 4 γ/ml; **2** = Östradiol 8 γ/ml; % = Verlängerung der Eier in % über den Normaldurchmesser. Jeder Punkt Mittelwert von 5 unabhängigen Bestimmungen.

Abb. 2 (*rechts*). Verhalten östradiolbehandelter unbefruchteter Eier und unbehandelter Eier nach Überführung in hypertonische Kochsalzlösung. *K* = unbehandelte Kontrolle; *Ö* = mit 4 γ/ml behandelte Eier; % = Anzahl von Eiern mit schrumpfiger Oberfläche in %.

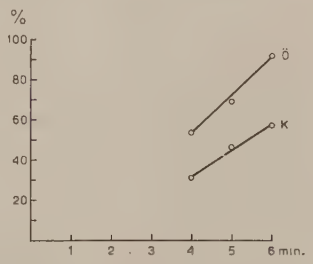


Abb. 3. Anzahl geschichteter Eier nach verschiedenen Zentrifugierungszeiten. *K* = unbehandelte Kontrolle; *Ö* = östradiolbehandelte Eier; % = Anzahl geschichteter Eier in %.

Ergebnisse. — Werden unbefruchtete Eier von *Psammechinus miliaris* 60 Minuten mit 4 γ/ml Östradiol behandelt, so zeigt sich beim Zentrifugieren, dass die Rigidität der Plasmamembran im Vergleich zu der unbehandelter Eier herabgesetzt ist. Diese Wirkung des Östradiols verstärkt sich bei Erhöhung der Konzentration auf 8 γ/ml, vgl. Abb. 1. In den Hypertonieversuchen wurde festgestellt, nach welcher Zeit die anfänglich auftretende Schrumpfigkeit der Oberfläche der Eier verschwindet. Abb. 2 zeigt, dass dieser Zustand bei östradiolbehandelten Eiern zeitiger eintritt als bei der Kontrolle. Dieser Befund steht mit dem Ergebnis der Zentrifugierversuche in Einklang.

Wie Abb. 3 zeigt, tritt die Schichtung der Plasmakomponenten bei östradiolbehandelten Eiern bei einer grösseren Anzahl und zeitiger auf als bei der unbehandelten Kontrolle.

Mit der gleichen Methode liess sich die Herabsetzung der Rigidität durch Östradiol auch bei befruchteten Eiern nachweisen, wenn die Eier nach der Befruchtung

Tabelle I

	Nich geteilte Eier %	Unregelmässig geteilte Eier %	Regelmässig geteilte Eier %	Totale Anzahl gezählter Eier
2 γ Östradiol per ml Seewasser	18	82	0	801
2 γ Östradiol per ml Seewasser mit 0.002 *M* ATP	10	32	58	526

zu einem Zeitpunkt zentrifugiert wurden, bei dem die normale Rigidität des Zytoplasmas steigt. Dieses ist kurz vor der Teilung der Eier, ca. 45 Min. nach der Befruchtung, der Fall. Wurden befruchtete Eier in 4 γ/ml Östradiol überführt, so zeigten sie, wenn sie nach 40 Minuten zwischen Seewasser und isotonischer Rohrzuckerlösung für 3 Min. zentrifugiert wurden, eine Anzahl von ca. 60 % geschichteten Eiern, gegenüber 20 % bei der unbehandelten Kontrolle. Ausserdem war die Schichtung der Plasmakomponenten bei den östradiolbehandelten Eiern ausgeprägter und fortgeschrittener als bei der Kontrolle.

ATP schützt in einem gewissen Grade die Eier gegen die Wirkung von Östradiol [7]. Ein Versuch wird hier angeführt:

Versuch 10.VII.54: Die Eier wurden in die Lösungen unmittelbar nach der Befruchtung übergeführt. Nach 4 Stunden erfolgte die Zählung der verschiedenen Klassen von Eiern. (Siehe Tabelle I.)

Besprechung der Ergebnisse. — Die von Agrell [1] hervorgehobene Ähnlichkeit zwischen der Wirkung von Östradiol und der von Colchicin bestätigt sich auch bezüglich der Einwirkung der betreffenden Substanzen auf die Rigidität des Zytoplasmas; betreffs weiterer Angaben über der Wirkung von Colchicin auf das Seeigelei vgl. [6]. Die unter der Wirkung von Östradiol eintretende Tendenz zur Lockerung der Zellen, besonders des vegetativen Bezirkes [7], kann mit einer Herabsetzung der Rigidität zusammenhängen. ATP wirkt offenbar dieser Lockerung entgegen.

Die vorliegende Untersuchung wurde von dem Schwedischen Cancer Verein und dem Hierta-Retzius Fond unterstützt.

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ZENTRIFUGIERVERSUCHE ÜBER DEN EINFLUSS VON PERJODAT AUF DEN ZUSTAND DER PLASMAMEMBRAN UND DES ZYTOPLASMAS DES SEEIGELEIES

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Die Wirkung von Perjodat auf die Eier von *Psammechinus miliaris* ist im Hinblick auf die Änderungen im Zustand der Plasma-membran und des Zytoplasmas in einer Reihe von früheren Arbeiten [2, 4, 6, 8] eingehend beschrieben worden. Die Ergebnisse dieser Arbeiten sind mit Hilfe von Zentrifugierversuchen gewonnen worden, bei denen sowohl die Streckung der Eier während des Zentrifugierens als auch die Schichtung der Plasmakomponenten verfolgt und bestimmt wurden. Es ist für die vorliegenden Untersuchungen von grundsätzlichem Interesse zu wissen, welchen Einfluss die Schnelligkeit und der Verlauf der Schichtung der Plasmakomponenten auf die während des Zentrifugierens beobachtete Streckung des Eies ausübt. Es könnte angenommen werden, dass die während des Zentrifugierens erfolgende Schichtung der Plasmakomponenten den Streckungszustand des Eies eindeutig bestimmt. Die unten mitgeteilten Versuche mit Eiern von *Psammechinus miliaris*, die mit Natriumperjodat behandelt wurden, zeigen aber, dass man zwischen Wirkungen auf die Plasmamembran und solchen auf das innere Zytoplasma (Endoplasma) unterscheiden kann.

Material und Methode. — Die vorliegende Untersuchung ist während der Sommermonate 1954–55 an der Zoologischen Station Kristineberg an der schwedischen Westküste durchgeführt worden.

Es wurden dafür Eier von *Psammechinus miliaris* verwendet, die bei dem für diese Art natürlichen Seewasser mit einem Salzgehalt von ca. 25 pro mille gehalten wurden. Die Gallerthülle der Eier wurde vorher entfernt. Für die Versuche wurde eine Natriumperjodatlösung von 5×10^{-5}-*N* verwendet, die jedesmal frisch zubereitet wurde. Nach entsprechender kurzer Perjodat-Behandlung von 3–5 Min. wurden die Eier in eine Mikroküvette übertragen und für die Dauer von 7 Minuten in einem Zentrifugenmikroskop (Fabrikat Struers, Kopenhagen) mit einer konstanten Zentrifugalkraft von 4350 *g* zentrifugiert. In der Küvette waren die Eier zwischen Seewasser und einer isotonischen Rohrzuckerlösung suspendiert. Die Streckung und Schichtung der Eier wurde während des Zentrifugierens mit einem Messokular beobachtet und mit der einer unbehandelten Kontrolle verglichen [2]. Um die Rigidität des Endoplasmas durch Messung der Schichtung der Plasmakomponenten exakter zu bestimmen, wurde das gleiche Material zwischen Seewasser und einer 20%igen reinen Gummiarabikum-Lösung von pH 8,2 in einer gewöhnlichen Zentrifuge bei Anwendung einer konstanten Zentrifugalkraft von 4460 *g* für die Dauer von 4–6 Minuten zentrifugiert. Hier erfolgt keine oder eine nur unbedeutende Streckung, und die Anzahl der geschichteten Eier lässt sich daher bequem bestimmen. Gleichzeitig mit den beschriebenen Untersuchungen wurde das Verhalten der Eier nach Überführung

in eine hypertonische Lösung beobachtet (0.6 ml einer 2.5 *N* NaCl Lösung + 2 ml Seewasser), um die dabei auftretende Form der Plasmolyse und die Anzahl der präaktivierten und aktivierten Eier festzustellen [2].

Ergebnisse. — Werden unbefruchtete Eier von *Psammechinus miliaris* kurzfristig (ca. 3 Min.) mit 5×10^{-5} *N* Natriumperjodat behandelt, so zeigen ca. 10–20 %

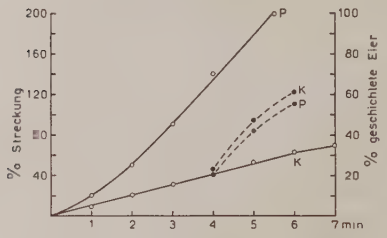

Abb. 1. Die voll gezogenen Linien geben die Streckung in Prozent vom Eidurchmesser an. Die gestrichelten Linien geben den Prozentsatz von geschichteten, nicht gestreckten Eiern an. *K*, Kontrolle in Seewasser; *P*, mit 5×10^{-5} *N* Natriumperjodat behandelte Eier. Jeder Punkt Mittelwert von drei unabhängigen Bestimmungen.

davon eine starke Streckung während des Zentrifugierens zwischen Seewasser und einer isotonischen Rohrzuckerlösung. Die Streckung erreicht schon nach 3 Min. den Wert von 90 % über den Normaldurchmesser. Es handelt sich in diesem Fall um sog. präaktivierte Eier, deren Plasmamembran sich infolge Perjodat-Behandlung in einem Zustand niedriger Rigidität befindet. Abb. 1 zeigt die Streckungskurve solcher Eier im Vergleich mit einer unbehandelten Kontrolle.

Auch die folgende Beobachtung ist in diesem Zusammenhang von Bedeutung. Mit Natriumperjodat vorbehandelte unbefruchtete Eier wurden im Zentrifugenmikroskop während drei Minuten zentrifugiert. Man erkannte sowohl stark verlängerte präaktivierte Eier als auch schwächer gestreckte Eier, die nicht aktiviert bzw. vollaktiviert sind. Die letzteren runden sich nach aufhören der Zentrifugierung im Laufe von 2–3 Minuten ab, während die ersteren während derselben Zeit deformiert bleiben. Dieses Verhalten spricht auch dafür, dass die Struktur der Plasmamembran bei den präaktivierten Eiern verändert ist.

Werden parallel mit 5×10^{-5} *N* Perjodat behandelte Eier und unbehandelte Eier zwischen Seewasser und Gummiarabikum-Lösung zentrifugiert, so zeigt sich in der Schichtung der Plasmakomponenten und in der Anzahl der geschichteten Eier kein wesentlicher Unterschied, wie aus Abb. (gestrichelte Kurve) hervorgeht. Aus der Kurve geht weiterhin hervor, dass die Anzahl geschichteter Eier nach einer Zentrifugierung von 4 Min. verhältnismässig niedrig ist und erst nach diesem Zeitpunkt ansteigt. Offenbar tritt die Schichtung erst mit einer gewissen Verzögerung ein, die wir früher abzuschätzen versucht haben [7].

Aus den geschilderten Ergebnissen lässt sich der Schluss ziehen, dass die starke Streckung präaktivierter, perjodatbehandelter Eier auf dem niedrigen Rigiditätszustand der Plasmamembran beruht und dass die Streckung nicht einfach von gleichzeitig sich ausbildenden sichtbaren Schichtungsvorgängen im Zytoplasma abhängig ist. Mit anderen Worten: die Methode erlaubt weitgehend, eine Veränderung der Rigidität der kortikalen Region unabhängig von der des Inneren zu bestimmen, was auch früher mitgeteilte Befunde wahrscheinlich machten [3].

In allen von mir untersuchten Fällen lief die erhöhte Streckung des Eies im Zentrifugalfeld mit einer herabgesetzten Schrumpfigkeit der Eier nach Überführung in

eine hypertonische Lösung einher. Wie bereits in einer früheren Arbeit [2] beschrieben, zeigen die durch Perjodat präaktivierten Eier von Anfang an eine glatte Oberfläche, während die nicht aktivierten Eier nach Überführng in die hypertonische Lösung eine Schrumpfung aufweisen [9], die einer höheren Rigidität entspricht. Die aktivierten Eier zeigen dagegen die eckige Plasmolyse, die einen Zustand noch höherer Rigidität der Oberflächenschicht des Eies angibt. Unter normalen Verhältnissen bildet sich dieser Zustand etwa fünf Minuten nach der Befruchtung aus, vgl. [3].

Nach längerer Perjodateinwirkung (45 Min.) wird die Anzahl der präaktivierten Eier geringer, während gleichzeitig die Anzahl der vollaktivierten Eier ansteigt. Das deutet darauf hin, dass der Zustand der Präaktivierung durch längere Perjodateinwirkung in den der Vollaktivierung übergeht. Die durch Perjodat vollaktivierten Eier unterscheiden sich in dem Zustand der Plasmamembran und der Rigidität des Endoplasmas nicht von normalen befruchteten Eiern.

Besprechung der Ergebnisse. — Die Ergebnisse der vorliegenden und früherer Arbeiten zeigen, dass die starke Streckung sog. präaktivierter Eier, die beim Zentrifugieren nach der Perjodatbehandlung beobachtet wird, auf Veränderungen beruht, die sich in der Plasmamembran des Eies vollzogen haben.

In dem Zustand des Endoplasmas präaktivierter Eier konnte dagegen gegenüber dem unbehandelter Eier kein Unterschied nachgewiesen werden. Weder bei der einen oder anderen Art lässt sich in den ersten Minuten während des Zentrifugierens eine ausgeprägte Schichtung der Endoplasmakomponenten nachweisen. Eine solche bildet sich erst allmählich aus, und zwar hauptsächlich in der Zeitspanne von 3 6 Minuten nach Beginn des Zentrifugierens. Vor dieser Periode hat sich die Streckung bei den präaktivierten Eiern schon weitgehend vollzogen.

Starke Streckungen der Eier im Zentrifugierversuch, die auf einer Senkung der Rigidität der Plasmamembran beruhen, treten u.a. bei Behandlung der Eier mit bestimmten Aminosäuren auf [2]. Auch in diesen Fällen erweist sich, dass der Zustand des Eies als Präaktivierung bezeichnet werden kann und dass diese Verbindungen die Befruchtungsfähigkeit der Eier verbessern.

Die starke Senkung der Rigidität der Plasmamembran, die bei den präaktivierten Eiern erfolgt, dürfte einer Depolymerisierung makromolekularer Komplexe entsprechen. Bei der vollen Aktivierung vollzieht sich eine Repolymerisierung. Ähnliche Umwandlungen treten wahrscheinlich auch als Folge normaler Befruchtung ein [5, 6].

Die Untersuchung wurde von dem Hierta-Retzius Fond und dem Schwedischen Cancer Verein unterstützt.

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CHANGES IN THE DNA CONTENT IN AN EHRLICH ASCITES TUMOR FROM 1952 TO 1956, AS DETERMINED BY FEULGEN MICROSPECTROPHOTOMETRY[1]

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In 1952, Leuchtenberger, Klein and Klein [3] reported the results of simultaneous DNA analyses on an Ehrlich ascites tumor grown in hybrid albino mice at the Karolinska Institute in Stockholm Sweden. A tetraploid DNA value was found by both ultraviolet microspectrophotometry and by chemical analysis. This value was

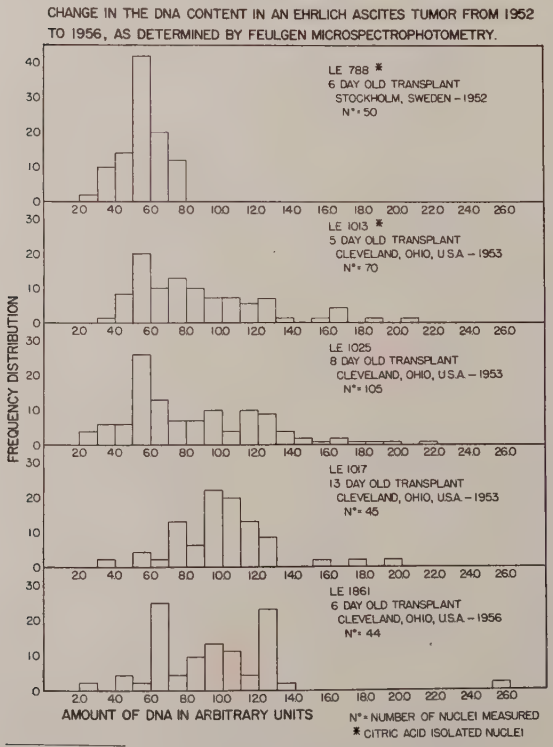
CHANGE IN THE DNA CONTENT IN AN EHRLICH ASCITES TUMOR FROM 1952 TO 1956, AS DETERMINED BY FEULGEN MICROSPECTROPHOTOMETRY.
LE 788 *
6 DAY OLD TRANSPLANT
STOCKHOLM, SWEDEN – 1952
N*= 50
LE 1013 *
5 DAY OLD TRANSPLANT
CLEVELAND, OHIO, U.S.A. – 1953
N*= 70
LE 1025
8 DAY OLD TRANSPLANT
CLEVELAND, OHIO, U.S.A. – 1953
N*= 105
LE 1017
13 DAY OLD TRANSPLANT
CLEVELAND, OHIO, U.S.A. – 1953
N*= 45
LE 1861
6 DAY OLD TRANSPLANT
CLEVELAND, OHIO, U.S.A. – 1956
N*= 44
FREQUENCY DISTRIBUTION
AMOUNT OF DNA IN ARBITRARY UNITS
N*= NUMBER OF NUCLEI MEASURED
* CITRIC ACID ISOLATED NUCLEI

Fig. 1.

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in agreement with an earlier biochemical value reported by Klein [2] and was also in accordance with the tetraploid number of chromosomes found by Hauschka and Levan [1]. Feulgen microspectrophotometric determinations on the same tumor sample confirmed the tetraploid DNA value as seen in the accompanying histogram (see Le 788).

However, Feulgen microspectrophotometric DNA analyses on the same tumor grown in hybrid albino mice in the United States showed different results from those obtained on the tumor grown in Sweden. From 1953 up to the present, each Ehrlich ascites tumor tested, showed a cell population with larger amounts of DNA. The accompanying histogram demonstrates that, in addition to the expected tetraploid values, many cells now contain significantly higher amounts of DNA, up to four times the tetraploid value.

It is tempting to attribute these changes either to a change in chromosomal number or to a change in mitotic activity. However, before such data become available, no explanation can be offered. Nevertheless, it does seem worthwhile to put these microspectrophotometric data on record.

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FURTHER STUDIES ON CROSS FERTILIZATION IN SEA URCHINS

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It was recently demonstrated that the jelly coat which surrounds the sea urchin egg acts as an inhibitor of fertilization [2, 5] and membrane elevation [11, 12]. Moreover, it was found that the jelly coat *in situ* protects the egg against polyspermy [3] and that eggs with intact jelly coats need more sperm to become fertilized to 100 per cent than do jelly-free eggs [3].

"Egg water", i.e. sea water containing dissolved jelly coat substance, has been claimed to act improving on cross fertilization to a certain extent [1, 7, 8, 9]. The present writer reinvestigated this action of egg water and was able to show that egg water acts quite unspecifically since it is possible to improve cross fertilization between two different species by adding egg water from a third species [4]. Furthermore, this improving influence of egg water can be replaced by low concentrations e.g. of heparin, which is chemically related to jelly coat substance [4, 10].

It is known that sperm motility is enhanced upon addition of jelly solution or heparin in appropriate concentrations and the present writer made it probable

TABLE I

Eggs from	Sperm from	Eggs with jelly coats, % fertilization	Eggs without jelly coats, % fertilization
Arbacia lixula	*Arbacia lixula*	100	100
,,	*Genocidaris maculata*	0	1
,,	*Paracentrotus lividus*	0	0
,,	*Psammechinus microtuberculatus*	0	0
Genocidaris maculata	*Genocidaris maculata*	100	100
,,	*Arbacia lixula*	5.7	44
,,	*Paracentrotus lividus*	50	57
,,	*Psammechinus microtuberculatus*	82	87
,,	*Sphaerechinus granularis*	9.1	17
Paracentrotus lividus	*Paracentrotus lividus*	100	100
,,	*Arbacia lixula*	0	14
,,	*Genocidaris maculata*	0	30
,,	*Psammechinus microtuberculatus*	4.8	24
Psammechinus microtuberculatus	*Psammechinus microtuberculatus*	100	100
,,	*Arbacia lixula*	0	0
,,	*Genocidaris maculata*	60	71
,,	*Paracentrotus lividus*	98	100

The concentration of sperm was 2×10^6 per ml throughout this experiment. The percentages of fertilization are based on counts of 200–250 eggs.

that the improving action of egg water in cross fertilization experiments is concerned with a species-nonspecific stimulation of the sperm (cf. [4]).

Vasseur investigated the chemical constitution of the jelly coat in several sea urchin species [13, 16, 17]. He demonstrated that considerable differences do exist in the composition of the carbohydrates of the jelly coat and also the amino acids of the jelly are different in the various sea urchin species [13, 16, 17]. Among others Vasseur [14, 15] also studied the specificity in sperm agglutination and succeeded in demonstrating that this phenomenon was fairly species-specific and could be used as a characteristic to distinguish between closely related species; for further references see [16].

More evidence in support of the view that the jelly coat *in situ* is a species-specific structure preventing cross fertilization was recently produced in experiments where the capacity for hybridization of eggs without jelly coats was compared with that of intact eggs [4, 6]. From these experiments it appeared that the jelly coat in some species represents a strong barrier to heterologous insemination (cf. [4]). However, in other species the removal of the jelly coat did not effect such a striking improvement in the percentage of cross fertilized eggs which indicates that in these species also other mechanisms do exist to prevent cross fertilization [4].

Material and Methods.—The experiments were made in Naples during March–May 1956 and the following species of sea urchins were used: *Arbacia lixula, Genocidaris maculata, Paracentrotus lividus, Psammechinus microtuberculatus and Sphaerechinus granularis.*

The jelly coats were removed by treating the eggs shortly in acid sea water at a pH of 5.6–5.8 (cf. [2]). The number of sperm was counted in a Bürker chamber after fixation in 3–4 per cent formaldehyde.

It is a general observation that more sperm is necessary to obtain cross fertilization than homologous fertilization. However, in this work insemination, homologous as well as heterologous, has been made at the same concentration of sperm to ensure that the numbers of cross fertilized eggs are comparable with the numbers of fertilized eggs in the controls. Moreover, the concentrations of sperm used have been 10^6–5×10^6 per ml and consequently the percentage of polyspermy in the controls has been very low.

Results.—In the present work some new crosses were made and the results support the view that the jelly coat *in situ* serves as a barrier to heterologous insemination. A difficulty which is met with in cross fertilization experiments is the necessity of a simultaneous supply of ripe gametes from a number of species, living on the same locality and spawning at the same time. This difficulty probably indicates that the need of a block to foreign spermatozoa is not very accentuated under natural conditions.

The results from a typical experiment are listed in Table I, and it is evident that in some of the crosses the percentage of hybrids is significantly increased upon removal of the jelly coats. Of particular interest is the crossing of *Genocidaris* female with *Arbacia* male because crosses with *Arbacia* sperm have always been regarded as difficult (cf. [1, 7, 8, 9]). The increase in the percentage of hybrids is very obvious upon removal of the jelly coats which is also the case in the cross *Paracentrotus* female *versus Genocidaris* male.

Cleavage and development are as a rule poor subsequent to cross fertilization and the hybrids with *Arbacia* sperm seldom reach the blastula stage. However, the crosses between *Genocidaris* and *Psammechinus* gave in both directions development to apparently normal free-swimming larvae.

The eggs of *Genocidaris* give very high fertilization membranes which was also found to be the case when the eggs were cross fertilized. The height of the fertilization membrane is, however, not only dependent upon the egg but also upon the sperm. This is indicated by the fact that *Psammechinus* eggs give, when inseminated with *Genocidaris* sperm, more highly elevated membranes than after insemination with *Psammechinus* sperm.

The eggs of *Genocidaris*, *Paracentrotus* and *Psammechinus* show a general, though sometimes rather weak, tendency to improved capacity for hybridization upon removal of the jelly coats. The *Arbacia* eggs on the other hand, give no such improvement which indicates that other factors than the jelly coat participate in preventing heterologous fertilization. It is obvious that the systematic relationship between the species tested must be of great importance to the capacity for cross fertilization and e.g. cytoplasmic factors might constitute a block to the entrance of heterologous spermatozoa. As a matter of fact *Arbacia lixula* stands in the system quite distant from the other sea urchins used in this work.

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